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(54) Title: TRANSPORT PEPTIDES AMONG WHICH C-TERMINAL Erns PEPTIDE AND ANALOGUES THEREOF

(57) Abstract: The invention relates to peptides derived from or similar to the Erns protein of pestiviruses for type -specific diagnosis of infection, for eliciting antibiotic activity and for transport of substances into a cell. For among others these purposes the invention provides an isolated, synthetic or recombinant protein module or functional equivalent thereof comprising an amino acid sequence that is at least 75 % identical to an amino acid sequence of a peptide located from at about amino acid position 194 to 220 in a pestiviral Erns protein.

Title:Transport peptides among which C-terminal E^{rns} peptide
and analogues thereof.

The invention relates to transport peptides, for example
derived from the E^{rns} protein of pestiviruses for type -
specific diagnosis of infection, for eliciting antibiotic
activity and for transport of substances into a cell.

5   Hog cholera virus or classical swine fever virus (CSFV),
bovine viral diarrhea virus (BVDV), and border disease virus
(BDV) belong to the genus *Pestivirus* of the *Flaviviridae*
family, CSFV is restricted to swine whereas BVDV and BDV have
been isolated from several species such as cattle, swine,
10   sheep, deer and giraffes Although pigs can be infected by
all these pestiviruses, only CSFV induces severe disease and
is often fatal. The disease is characterized by fever and for
instance leukopenia and can run an acute, chronic or
subclinical course. Although effective live-attenuated
15   vaccines are available, pigs are not vaccinated against
CSFV in the European Union because vaccinated and infected
pigs are serologically indistinguishable. Outbreaks of CSF in
the EU are controlled by eradication of all pigs from
infected farms and farms in the vicinity. Because of this
20   strategy, more than 10 million pigs had to be killed and
destroyed during the 1997-1998 CSF epizootic in The
Netherlands costing more than 2 billion US dollars. It is for
this reason that their is a great demand for a marker vaccine
which can provide protective immunity and which induces an
25   antibody response in the vaccinated pigs which can be
distinguished from the antibody response caused by a natural
CSFV infection.
Like other members of the family, pestiviruses are plus-
stranded RNA viruses whose genome comprises one long open
30   reading frame. Translation into a hypothetical polyprotein is
accompanied by processing into mature proteins. The
structural proteins include a nucleocapsid protein C and
three envelope glycoproteins E^{rns}, E1 and E2. The envelope
proteins E^{rns} and E2 are able to induce neutralizing

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antibodies. Glycoprotein E2 is the most immunogenic protein
of pestiviruses and elicits high titers of neutralizing
antibodies after infection. Vaccination of target animals
with E2 has shown to give complete protection against a

5   lethal homologous challenge. When E2 is used for vaccination,
serological diagnosis of a natural pestivirus infection has
to be performed with an immunogenic/antigenic protein other
than E2 that is present in the infectious pestivirus. For
this purpose the E^{rns} glycoprotein can be used as an antigen

10   in a diagnostic test. A population that is vaccinated with
the E2 glycoprotein can still be tested serologically for
pestivirus infection with a diagnostic test based on the E^{rns}
antigen. A serological test based on E^{rns} can distinguish E^{rns}-
antibody positive sera from animals infected with the virus

15   and E^{rns}-antibody negative sera from uninfected animals. This
is called the marker vaccine approach. Of course these
markervaccins depend on sensitive tests and in the case of
CSFV, the test also has to be very specific because pigs can
be infected with the other pestiviruses BVDV and BDV. Because

20   BVDV and BDV do not cause (severe) clinical symptoms in pigs
and the animals are not vaccinated for these viruses, the
diagnostic test for a CSFV marker vaccine should only detect
CSFV antibodies and no other pestivirus antibodies.
Serological tests based on the complete E^{rns} protein have been

25   developed previously but are not always satisfactory in that
they are not specific enough in that they cannot discriminate
sufficiently between infections with different pestivirus
species or are not sensitive enough to detect early
infections with a pestivirus.

30

In one embodiment, the invention provides a so-called
transport peptide module, herein also called movin. In
principle, we found that most linear peptides of 10 to 18
residues long which have > 40% arginines (R) or lysines (K)

35   are capable of functioning as such a transport peptide module
to which cargo can be attached. Such a transport peptide

module preferably should not or only to a small extent
contain negatively charged amino acids such as aspartic acid
(D) or glutamic acid (E). Preferred peptide modules are
identified herein with full sequence, such as for example in

5   tables 4, 5, and 9 to 11, or retro-inverso variants thereof.
Variations in amino acid sequence are well tolerated, at
least from the viewpoint of translocation activity, as a rule
of thumb it can be said that related sequences have at least
30-50% homology, preferably at least 70% homology, most

10   preferably at least 85% homology, to those displayed in said
tables, which allows identifying further relevant sequences
present in nature or capable of being synthesised.
Substitutions in the amino acid sequence of a transport
peptide module can be applied to increase the translocation

15   (transport) activity. An optimized transport peptide module
can for example be synthesized according to retro-inverso
peptide chemistry, in which the sequence is reversed and D-
amino acids are used instead of L-amino acids. Transport
peptides derived from the herein indicated positions of Erns

20   peptide, L3 loop peptides or human respiratory syncytial
virus protein G (HRSV-G) peptides, and peptide mimics or
peptoides derived thereof were able to bind surface
glycosaminoglycans like heparin. Therefore, finding that a
peptide belongs to the group of linear heparin binding

25   peptides or is capable of binding related glycosaminoglycans
can be used as a prediction that they likely also can
function as transport peptides. However, heparin binding is
not a prerequisite for a peptide being a transport peptide.
To check if the presence of heparin on the surface of the

30   cell influenced the efficiency of translocation, it was
tested whether heparin-binding peptides also translocated
into mutant cells which were glycosaminoglycan deficient
(cell lines pgsA-745 and pgsD-677). Titrations of all
heparin-binding peptides on the different cells showed that

35   peptides translocated with the same efficiency/activity into
heparin containing cells and in the mutant cells without

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heparin (data not shown). Thus, heparin-binding peptides have
translocation activity and binding of the peptides to
heparin, obviously does not block the peptide from
penetrating the plasma membrane. Likely, the peptides have a
5   high on/off rate for heparin and the high affinity for
fosfolipids directs the peptides to the membrane and
ultimately into the cell. On the other hand, heparin binding,
albeit being predictive, does not seem to be a prerequisite
for efficient translocation of the peptides.
10   The invention further provides a method for translocating a
compound over a membrane of a cell, an epithelial layer,
mucus layer, blood - brain barrier or skin comprising
providing said compound with a transport peptide module
according to the invention and contacting it with a cell.
15   Such compounds, herein also called cargo, can be large,
successful translocation of compounds up to 600 kD has been
demonstrated and it is ecpected that even larger compounds
may be translocated. From the perspective of speed of
translaoction in relation to the usefulness of the compound,
20   compounds of preferred molecular weight are those of 60 to
500 kD and even more preferred are those of 120 to 300 kD.
Compounds can also be of a varied nature, for example, it is
possible to link macromolecules such as nucleotides,
polypeptides, drugs such as antiviral, antimicrobial or anti-
25   inflammatory drugs, and the like to a module as provided
herein for successful translocation of such a compound.
Topical application of such a compound, e.g. as a
pharmaceutical composition, is specifically provided, a
module as provided herein has excellent capacity to penetrate
30   to the upper layers of the skin. Typical applications include
further use of a labile linker such as a thioester or a
labile linker such as a $O(C=O)CH_2NRC(=O)CH_2NHCH_2(C=O)SCys$
linker. For use of a transport peptide module according to
the invention, drugs or macromolecules are typically
35   covalently coupled to said peptide, examples are cyclosporine

5

A , acyclovir , and terbenafine coupled with a module
according to the invention.

This invention also provides among others peptide-based
diagnostics in connection with diseases caused by pestivirus

5   infections. Antigenic peptides as provided herein and useful
for diagnostics can surprisingly also be used otherwise, such
as antibacterial or transport peptides. Because in one
embodiment the transport peptide module is a fragment derived
of the E^{rns} protein, it can be used for diagnosis of

10   pestivirus infections when a markervaccin is used that is
based on E2, another pestivirus surface protein. Due to its
unique biochemical character, a peptide as provided herein
has the ability to permeate and kill micro-organisms and has
the ability to translocate itself and a coupled cargo across

15   a cell membrane and epithelium barrier.
In a preferred embodiment, the invention provides a thus far
unidentified small, independently folding protein (peptide)
module related to modules present at the C-terminal end of
pestivirus E^{rns}, at the L3 loop of secreted cytotoxic Rnases

20   that preferably belong to the group of type II ribotoxins
such as alpha-sarcin, restictocin, mitogillin, toxin Asp fI,
clavin or gigantin, in a heparine-binding peptide, a DNA/RNA
binding peptide, in HRSV-G protein, and its use as a
transport peptide. Previously, the region responsible for

25   translocation of alpha-sarcin was thought to be located in a
hydrophobic stretch, located away from the L3 loop (Mancheno
et al., Biophys. J. 68, 2387-2395, 1995. In a preferred
embodiment, the invention provides an isolated, synthetic or
recombinant protein module or functional equivalent thereof

30   comprising an amino acid sequence that is at least 85%
identical to any of the sequences shown in tables 1-4 and 9-
11, e.g., to an amino acid sequence of a peptide located from
at about amino acid position 194 to 220 in a pestiviral E^{rns}
protein and/or that is at least 70% identical to a L3 loop

35   sequence such as shown in table 5.

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Such transport peptide modules can be prepared synthetically
with normal peptide synthesis or coupling techniques as
described herein starting from individual amino acids or by
coupling or linking smaller peptides of relevant sequence to
another or by cleaving off from larger peptides. When
desired, non-conventional amino acids can be used such as D-
amino acids or others that normally do not occur in natural
proteins. Peptides can also be prepared via recombinant DNA-
techniques via transcription and translation from recombinant
nucleic acid encoding such a peptide or protein module, be it
linked to for example a fusion protein or specific target
molecule such as a desired binding molecule derived from an
antibody or protein ligand or receptor binding molecule, and
so on. For example, we have successfully expressed a fusion
protein of a transportpeptide and Green Fluorescent protein
in A72 cells. The Green Fluorescent protein showed the same
cellular localisation as the biotinylated transportpeptide:
nucleoli, and around the nucleus. This in contrast to
normally expressed Green Fluorescent protein which was
distributed evenly over the cell (data not shown).

In a preferred embodiment, the invention provides a
transport peptide module or functional part thereof wherein
at least said functional part of said peptide comprises a
reversed amino acid sequence to one of a sequence given in
claim 1 to 6 and wherein D-amino acids are used instead of L-
amino acids. Reversing the sequence, and using the D-amino
acids instead enhances translocation activity, allowing
improved use for for example transport of macromolecules or
drugs through cell membrane barriers into cells.
In a preferred embodiment as explained herein, the invention
provides a module which is functional as transport peptide
module, also when cargo is attached, wherein said peptide is
located from at about amino acid position 191 to 222, or from
about 194 to 227, or from about 191 to 227, or from at about
amino acid position 176 to about 220, 222, or 227 in the case
of the pestiviral E^{rns} protein or residues 51-91 or ~~53~~ 59-88

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or from 62 - 88 or from 62 - 74, in the case of the L3 loop
protein, or from at about amino acid position 187 to 223 in a
respiratory syncytial virus G-protein. Also, in HRSV type B.
a similar region was detected from position 149 to 160 in
5   protein G. These amino acid positions and their numbering are
of course relative to known sequences as for example shown in
the figures herein wherein alignments of various pestiviral
sequences are shown, which of course allows for example for
alignment with yet unknown pestiviral sequences, and allows
10 alignment with ribotoxin L3 loop sequences. As a rule of
thumb it can be said that related sequences have at least 30-
50% homology, preferably at least 70% homology, most
preferably at least 85% homology, which allows identifying
further relevant sequences present in nature or capable of
15 being synthesised. As examples herein modules are described
wherein said peptide comprises the amino acid sequence
RQGAARVTSW LGRQLRIAGK RLEGRSK or RQGTAKLTTW LGKQLGILGK
KLENKSK or RVGTAKLTTW LGKQLGILGK KLENKTK or RQGAAKLTSW
LGKQLGIMGK KLEHKSK, or GNGKLIKGRTPIKFGKADCD RPPKHSQNGMGK or
20 GDGKLIPGRTPIKFGKSDCDRPPKHSKDGNGK or
GEGKILKGRTPIKFGKSDCDRPPKHSKDGNGK or GDGKILKGRTPIK
WGNSDCDRPPKHSKNGDGK or KRIPNKKPGKK or KTIPSNKPKKK or
KPRSKNPPKKPK or a functional part thereof however, variations
can be introduced for example by increasing the positive
25 charge of the peptide, preferably at positions that optimise
the amphipathic nature of the peptide, but not necessarily.
Another example is changing several or all L-amino acids to
D-amino acids to reduce possible protease sensitivity. The
translocation activity of the E^{rns} peptide was further
30 improved by substitution of the 2 lysines and the glutaminic
acid by arginines. In a preferred embodiment, a retro-inverso
variant of an above identified peptide module is provided;
such a retro-inverso peptide with an inversed sequence and D-
amino acids replacing L-amino acids comprises even higher
35 translocation activity.

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Of course, the invention also provides a recombinant nucleic
acid encoding a module according to the invention, for
example to provide for a proteinaceous substance provided
with a module according to the invention, for example
5 provided with a targeting means.
The invention in one aspect also relates to the design of an
antigenic substance, preferably peptide-based, corresponding
to said protein module in the E^{rns} protein of Pestiviruses or
a L3 loop of ribotoxin II can be used as a basis for e.g.
10 diagnostics tests, antibacterial or transporter peptides. For
example, in one embodiment, the invention provides a method
for inducing an antibody comprising administering to a host
capable of forming antibodies a module or a substance
according to the invention. Antibodies can be induced
15 classically, by for example immunising an animal with said
antigenic substance, or via more modern techniques, such as
phage display, whereby so called synthetic antibodies are
produced. Be it synthetic or classical (mono- or polyclonal),
the invention provides an antibody specifically directed
20 against a module according to the invention.
With the pestivirus-derived module and/or the antibody as
provided herein, the invention provides a method for
detecting the presence or absence of an antibody directed
against a pestivirus in a sample comprising contacting said
25 sample with a module or a substance according to the
invention said method preferably further comprising detecting
the presence or absence of an antibody bound to said module
or substance. Also is provided a method further comprising
contacting said sample with said module or substance in the
30 presence of a competing antibody directed against said module
and detecting the presence or absence of competing antibody
bound to said module or substance. Herewith the invention
provides use of a method according to the invention for
differentiating at least one animal from at least another
35 animal. The invention thus provides a test which is based on
a small fragment of the E^{rns} protein. Sequence analysis and

homology modelling was used for pestivirus E^{rns} to identify a region that can be used for the design of antigenic substances and resulted in the identification of a small independently folding protein module which in its native
5   state is exposed on the protein surface of the complete E^{rns} protein, can be used to design antigenic substances which are comparable or superior to the complete protein.
In a further embodiment the invention not only provides a peptide that behaves as a superior antigen in the E^{rns}
10   peptide-Elisa but one that has additional characteristics that are very interesting and useful. Due to its unique biochemical nature a peptide as provided herein, for example corresponding to the E^{rns} C-terminal domain or to a L3 loop in a ribotoxin, is able to interact with a cell membrane and
15   destabilize the membrane.
The invention further provides a method for translocating a compound over a membrane of a cell, an epithelial layer, mucus layer, blood - brain barrier or skin comprising providing said compound with a module or substance or
20   transport peptide module according to the invention and contacting it with a cell, and, furthermore, it provides a method for eliciting antibiotic activity to a micro-organism comprising contacting said micro-organism with said module or substance.
25   Herein it is shown that such an E^{rns} peptide or protein module as provided herein has antibacterial activity for for example gram-negative bacteria ($E.$ $Coli$) and an L3 loop or E^{rns} peptide it has translocation activity for for example eukaryotic cell membranes. A biological membrane is a very
30   efficient barrier that protects the micromilieu of cells or intracellular compartments from the outside milieu. In order to interfere directly with biological processes inside the cell, it is necessary that pharmaceuticals cross the lipid bilayer to block/bind their targets. Many promising,
35   potential therapeutics (hydrophilic organic molecules, peptides, proteins or genes) are ineffective because the cell

membrane forms an insurmountable barrier. However, several
peptides have been discovered recently, that can solve this
problem because they are able to translocate over the lipid
bilayer and are also able to transport a diverse set of
cargoes inside the cell.

Interactions of pore forming peptides with model and
artificial membranes have been studied extensively the last
three decades. Several families of membrane destabilising
peptides with antitumor, haemolytic, antibacterial activity
or a combination have been found. Many of these peptides form
amphipathic helices with a hydrophobic face and a positive
charged face that organise and aggregate on the membrane
surface and destabilise the membrane. Their mode of action
has some resemblance with the recently discovered transport
peptides (Matsuzaki et al, Biochim Biophys Acta 1376: 391-
400, 1998; Lindgren et al., Trends Pharmacol SCI 21: 99-103,
2000). The invention now provides a pharmaceutical
composition comprising a module or substance according to the
invention useful for several purposes. For example, the
invention provides use of a module or of a substance
according to the invention for the preparation of a
pharmaceutical composition capable of membrane translocation
(a transport peptide), for the preparation of a
pharmaceutical composition capable of eliciting antibiotic
activity (an antibiotic), or for the preparation of a
pharmaceutical composition capable of inducing antibodies(a
vaccine) upon administration to a host.

The invention is further explained in the detailed
description described herein without limiting it thereto.

Detailed description
For diagnostics, the invention provides a peptide comprising
an amino acid sequence derived from E^{rns} of a pestivirus,
wherein said amino acid sequence has a for example length of
37 amino acid residues corresponding to the C-terminus of
E^{rns}, which is located C-terminal to the RNase domain.
Preferably, said amino acid sequence comprises at least the
amino acid residues 191 - 227 of Pestivirus E^{rns} and has at
most 4 amino acid differences therewith. Preferably, said
pestivirus E^{rns} peptide is selected from the group consisting
of Classical Swine Fever Virus (CSFV), strain Alfort 187,
BVDV-1 strain M96751, BVDV-2, or BDV, strain X818. Said amino
acid sequence preferably comprises a member selected from the
group consisting of:

Table 1:

CSFV	: ENARQGAARV	TSWLGRQLRI	AGKRLEGRSK	TW,
BVDV-1	: EGARQGTAKL	TTWLGKQLGI	LGKKLENKSK	TW,
BVDV-2	: EGARVGTAKL	TTWLGKQLGI	LGKKLENKTK	AW,
BDV	: ENARQGAAKL	TSWLGKQLGI	MGKKLEHKSK	TW;

More preferably a member selected from the group consisting
of

Table 2:

CSFV	: ENARQGAARV	TSWLGRQLRI	AGKRLEGRSK	TWFGAYA,
BVDV-1	: EGARQGTAKL	TTWLGKQLGI	LGKKLENKSK	TWFGAYA,
BVDV-2	: EGARVGTAKL	TTWLGKQLGI	LGKKLENKTK	AWFGAHA,
BDV	: ENARQGAAKL	TSWLGKQLGI	MGKKLEHKSK	TWFGANA;

Such as a member selected from the group consisting of

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Table 3:
CSFV   DTALYLVDGMTNTI ENARQGAARV TSWLGRQLRI AGKRLEGRSK
TWFGAYA

BVDV-1 .   DTTLYLVDGLTNSL EGARQGTAKL TTWLGKQLGI LGKKLENKSK
TWFGAYA

BVDV-2   ETAIQLLDGATNTI EGARVGTAKL TTWLGKQLGI LGKKLENKTK
AWFGAHA

BDV   DTALYVVDGVTNTV ENARQGAAKL TSWLGKQLGI MGKKLEHKSK
TWFGANA

It is preferable that the peptide is capable of adopting the
tertiary structure of its counterpart in the corresponding
E^{rns} protein when it relates to an antigenic substance, or a
precursor thereof, which allows discrimination between or
identification of different pestivirus types or subtypes, or
allows discrimination between or identification of antibodies
against different pestivirus types or subtypes, which
antigenic substance or precursor thereof comprises a peptide
as defined herein.

A peptide, antigenic substance or precursor thereof as
defined herein may be used in diagnosis of Pestivirus
infections. This invention also provides a diagnostic testkit
for the detection of Pestivirus, or antibodies against
Pestivirus types or subtypes, which testkit comprises a
peptide, antigenic substance or precursor thereof as defined
herein, together with suitable means for detection. The
testkit preferably provides for an enzyme linked
immunosorbent assay.

The invention also provides a method for the detection of
(antibodies against) Pestivirus comprising contacting a
sample of a body fluid with a peptide, antigenic substance or
precursor thereof as defined herein, in a manner such that a
complex comprising said peptide, antigenic substance or
precursor, and an antibody directed against said peptide,
substance or precursor can be formed, followed by detection
of said complex.

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Furthermore, the invention provides a pharmaceutical
composition or vaccine for the prophylaxis of Pestivirus
infections comprising a peptide, antigenic substance or
precursor thereof as defined herein, together with a suitable
5   adjuvant or excipient for administration to a mammal.
The invention also provides a method for the prophylaxis of
Pestivirus infections comprising administering to a mammal a
composition as defined above, in an amount sufficient to
elicit an immune response against Pestivirus.
10   Furthermore, the invention provides a peptidomimeticum that
mimics a peptide as defined herein.
Another aspect of this invention is a method for inducing
antibodies against Pestivirus types or subtypes comprising
administering to a mammalian host an antigenic substance or
15   precursor thereof as defined herein, together with a suitable
adjuvant and harvesting resulting antibodies or antibody
producing cells from said mammalian host.
An antibody directed against a type or subtype of Pestivirus
obtainable by the above method is also part of the invention.
20   Preferably, the antibody is a monoclonal antibody.
In another aspect, the invention provides a diagnostic
testkit for the detection of or the discrimination between
(antibodies against) subtypes or types of Pestivirus
comprising the above antibody and suitable means for
25   detection.
For antibacterial and transport activity, the invention
provides a similar amino acid sequence as listed in table 1
and 2. Systematic analysis showed that shorter peptides
comprising E^{rns} amino acids 194 - 220 had higher transport
30   activity and lower hemolytic activity. Said amino acid
sequence preferably comprises a member selected from the
group consisting of:

35

Table 4:

CSFV   : RQGAARVTSW LGRQLRIAGK RLEGRSK,
BVDV-1   : RQGTAKLTTW LGKQLGILGK KLENKSK,
BVDV-2   : RVGTAKLTTW LGKQLGILGK KLENKTK,
5   BDV   : RQGAAKLTSW LGKQLGIMGK KLEHKSK;

or those presented in Table 5 relating to the L3 loop of
ribosome-inactivating proteins.

10   Most peptides that have been used in serology represent
continuous epitopes. It is impossible to detect antibodies
against complex discontinuous epitopes using small linear
peptides and it is difficult to predict discontinuous
epitopes based on the amino acid sequence of a protein. In
15   addition, the antigenic surface of large globular proteins
cannot be mimicked accurately with a small linear peptide. We
solved this problem by predicting an independently folding
region in the E^{rns} protein of pestiviruses that adopts a
stable tertiary structure while retaining its antigenicity.
20   This prediction is crucial for the correct design of a useful
antigen. Two stretches of Pestivirus E^{rns} show sequence
homology with ribonuclease Rh (RNase Rh), a new class of
microbial ribonuclease of *Rhizopus niveus*, member of the T_2/S
RNase superfamily. A typical feature for this type of RNase
25   is the low base specificity and the large molecular weight.
The crystal structure of RNase Rh has been determined
(Kurihara et al., J. Mol Biol 255: 310-320, 1996) and the
three-dimensional (3D) structure confirmed that both
stretches with sequence homology to E^{rns} constitute the active
30   site of the RNase. Apart from the two stretches of sequence
homology, further homology in the rest of the protein was not
apparent.
Despite a low sequence homology , we were able to construct
an alignment using different types of scoring matrices and
35   multiple sequence alignment of a large set of RNase
sequences. A satisfactory alignment was not possible using

alignment software with any parameter setting. Therefore, a
part of the alignment was edited manually. For parts with low
sequence homology, the alignment was guided by secondary
structure prediction of the PHD software (Rost, B., and C.
5   Sander. 1992. Nature. 360:540.)
After inspection of the multiple sequence alignment, some
major dissimilarities between the sequences of pestivirus E^{rns}
and the other RNases can be observed. Compared with sequences
of the other RNases, the pestivirus sequences have a
10   truncation at the N-terminus, large insertions after residue
83 and 135 and an elongated and very dissimilar C-terminus.
The 37 C-terminal residues could not be aligned with the
other RNases. Other characteristics of the C-terminus are the
high number of positive charges and a high score for
15   amphipathic helicity. A helical wheel representation of
residues 191 - 221 shows an amphipathic helix with a
hydrophobic face and a positively charged face. (Figure 2).
The only three residues that may not correspond with the
perfect amphipaticity are Ile210, Arg214 and Arg218.
20   Although no obvious domains were found with software like
SMART (Schultz et al., PNAS 95: 5857-5864, 1998), this C-
terminal region which is separate from the RNase domain
according to the alignment and with it's typical secondary
structure can now be considered as a separate domain or
25   module. Such a positively charged domain in an RNase molecule
is not unique for E rns but has also been observed in type II
ribotoxins, another class of RNases. This class of RNases are
extracellular cytotoxins that hydrolyse the large ribosomal
RNA (22) and are able to translocate across phopholipid
30   bilayers (23). Although ribotoxins are known to enter cells,
it is not known which region of the protein is responsible
for translocation. The type II ribotoxins like alpha-sarcin
and restrictocin contain a large inserted L3 loop (residue 53
- 91) compared with other RNases of the T1 superfamily
35   (24,25). This loop has structural similarity (but no sequence
similarity) to loops found in lectin sugar-binding domains

and may be responsible for the ribotoxins ability to bind the cell

surface (24). The C-terminal domain of E rns has approximately the same length and contains similar sequence
5   motifs as the ribotoxin II L3 loop (Figure 3). Although the sequence similarity between the ribotoxin L3 loop and the C-terminus of E rns is low (Figure 3), it is higher than the sequence similarity between L3 and the structurally similar lectin binding domains (24). Although the ribotoxin L3 loop
10 is also positively charged, it has no apparent amphipathic character. Another interesting homology of the E rns C-terminal region is with the membrane interacting peptide magainin. The center of the E rns peptide has high sequence homology with the N-terminal half of magainin (Figure 3).
15 This homology is even higher compared to the homology of magainin with other pore forming peptides that have been described (26)

The (overall) 3D structure of E^{rns} is similar to RNase Rh
20 except for the C-terminal region, which is surprisingly similar to loop L3 of restrictocin or other ribotoxin II proteins. (This protein module likely folds independently and is metastable and can change to an alpha helical structure when it binds the cell membrane). The 3D structure of the C-
25 terminal domain is not very important because of its spatial independence from the RNase domain.
        With the aid of a modular structure it is possible to define antigenic regions on the surface of the protein which can be mimicked by single linear peptides. The domain
30 corresponding to the C-terminal 37 residues (191 - 227) is the best candidate because of its location on the outer rim on the surface of the E^{rns} dimer, it forms a small functional domain which folds independently from the rest of the protein and it is not masked by any potential carbohydrate.
35

17

Development Elisa

The invention provides an antigenic and in essence
proteinaceous substance for discrimination of infected
animals with different types of pestiviruses from animals

5   vaccinated with a subunit vaccine that does not contain the
E^{rns} peptide and infected animals. The antigenic substance is
a peptide that corresponds to a C-terminal amino acid
sequence of Pestivirus E^{rns} which does not align with RNase Rh
but with an L3 loop of a ribotoxin and folds independently

10   from the rest of the protein.

An antigenic substance according to this invention is to be
interpreted as any peptide-like or peptide-based substance
(e.g. a protein module as provided herein optionally linked
to a another group such as a peptide or protein) capable of

15   inducing an immune response against pestivirus or being
recognized by a serum containing antibodies against a
pestivirus. Precursors of such antigenic substances are for
example comparable peptide-like or peptide-based substances,
which are not immunogenic themselves but need for instance to

20   be coupled to a carrier to be able to induce an immune
response or to be recognized. Peptide-based or peptide-like
substances are intended to include anything with the function
of the peptides according to the present invention. This
means that these substances may be peptides themselves in

25   which a number of amino acid residues have been replaced or
modified. It also means that they may be fusion proteins for
instance designed to present the amino acid sequence of the
peptides of the invention on their surface. The definition
also includes peptidomimetics and anti-idiotype antibodies

30   derived from the peptides according to the invention.

In a preferred embodiment the invention provides peptides
that can be used in diagnostic assays for detection of
antibodies directed against specific pestivirus types (CSFV,
BDV, BVDV-I, BVDV-II).

35   The provision of the protein module, the independently
folding region of E^{rns}, relates to all types of pestiviruses,

and beyond. As a consequence, the invention is not limited to
the peptides specifically disclosed herein, but extends to
analogous peptides and their derivatives in all types of
pestiviruses and all subtypes of these viruses and to
5 homologues of the L3 loop of (ribosome inactivating)
ribotoxin II proteins. Preferred peptides to be used
according to the invention comprise at least antigenic parts
of the peptides given in table 2 or derivatives thereof,
their length being from about 27 residues up to about 51
10 residues or transport peptides according to anyone of tables
1 - 5.
We have evaluated the applicability of the peptides in
diagnostics by the development of different diagnostic
assays: indirect ELISAs in which the antigen is recognized in
15 solid phase, an indirect ELISA in which the antigen is
recognized in liquid phase. Other diagnostic assays can of
course be easily designed by the man skilled in the art.
These may of course be provided in any suitable format.
Assays can be performed in solution and on solid phases, they
20 can be performed using any kind of label, such as enzymes,
solid particles, such as metal sols, or other sols, latex
particles, dyes, fluorescent substances or radioactive
materials. They even may be performed without labels, as can
be done by agglutination assays. The peptides can be used to
25 detect antibodies in for instance a fluid from a mammal, such
as blood, serum, urine, and milk. Usually the antibody is
bound by a peptide, module or substance according to the
invention, which may be present on a solid phase or in a
liquid phase. Afterwards the complex of peptide and antibody
30 may be detected by a labelled reagent, which can be a
labelled antibody directed against the host's (such as swine,
bovine or sheep) antibodies.
According to the invention the peptides can also be used to
obtain antibodies which are specific for Pestivirus types
35 and/or subtypes. The peptides are administered to a mammal,
usually a rodent, in an immunogenic form and after one or

more booster administrations the serum from the animal is
harvested and antibodies can be purified therefrom.
Alternatively, the spleen of such animals may be removed to
obtain antibody-producing cells. These can be changed, by
5   fusion or transformation, into cell lines producing
monoclonal antibodies. Assays based on (monoclonal)
antibodies directed Pestivirus and induced by the peptides
according to the invention are therefore also a part of the
invention.

10   The peptides according to the invention can of course also be
used in vaccines to prevent infection with Pestivirus. They
may be used in conjunction with other antigens to elicit an
immune response against Pestivirus. Usually the peptide has
to be coupled to a carrier to be presented in an immunogenic

15   form before administration to a host. Other ways of rendering
a peptide sufficiently immunogenic are known to the person
skilled in the art. Adjuvants are usually added to vaccines
to boost the immune response in a more aspecific manner.

20   Antibacterial and transport peptide
The invention further provides a membrane active peptide or
module or substance which can be used as antibiotic and can
be used as transport peptide which is for example capable to
carry cargoes across the cell membrane. For coupling of cargo

25   such as macromolecules, such as drugs, use is preferably made
of a free hydroxyl group, when available on the compound to
be bound.
To use transport peptides as drug-delivery system these
peptides can be linked to drugs via linkers. From the side of

30   the drug or compound, for instance cyclosporine A, acyclovir,
or terbenafine, a functional group like the hydroxyl group
can be used to couple a linking group via an ester bond. In
the linking group a function like a secondary amine (-NH-)
can be inserted, which, by its orientation catalyses the

35   cleavage of the ester bond with the drug and subsequently
releases the original drug. An example of such a

20

linker is: (drug)-O-CO-CH2-NR-CO-CH2-NH-CH2-CO-link2-
(transport peptide).
The transport peptide can be coupled to this linker by a
second linker (link2) using different chemistries. For
5   instance, ethylenediamine can be coupled to the free
carboxylic acid of the first linker. Subsequently, the
resulting aminogroup can be coupled to a bromoacetic acid and
this bromoacetylgroup can react in high yield with, for
instance the free -SH group of a cysteine in the transporter
10   peptide. Alternatively, the transport peptide can also be
coupled directly to the labile linker via for instance a
free aminogroup from the transport peptide.
Substituents in the linker on the different groups, like the
group R on the tertiairy amine, can help to regulate further
15   the stability of the ester. Alternatively, thioesters can
also be used as an even more labile linking group.
Alternative strategies can be used for the coupling of the
transporter peptide to compounds that have no easily
accessible hydroxyl group available. In the case of
20   terbenafine, an ethynyletheen function can for example be
used for the addition of the free -SH group in the linker to
form for instance:
(drug)-S-(CH2)n-NR-CO-CH2-NH-CH2-CO-link2-(transport
peptide). This conjugate can easily be cleaved under basic
25   condition (internal base is present as secundary NH-group),
releasing the original druh, here in the example terbenafine.
The membrane active peptide is similar to the described
antigenic peptide which corresponds to a C-terminal amino
acid sequence of Pestivirus E^{rns} which does not align with
30   RNase Rh but does align with magainin and to some extent with
the L3 loop of ribotoxins and folds independently from the
rest of the protein. The L3 loop peptides are specifically
membrane active peptides according to the invention as well.
A membrane active substance according to this invention
35   comprises for example a peptide-like or peptide-based
substance capable of inducing leakage of a bacterial membrane

or disturbance of an eukaryotic cell membrane without
leakage. Peptide-based or peptide-like substances are
intended to include anything with the function of the
peptides according to the present invention. This means that
5   these substances may be peptides themselves in which a number
of amino acid residues have been replaced or modified. It
also means that they may be fusion proteins for instance
designed to modify the cell specificity of the peptide.
Preferred peptides to be used according to the invention
10 comprise at least the membrane active part of the peptides
given in table 4, 5 and 10. Derivatives with a higher
translocation activity are listed in table 9.
The invention relates to a set of pestivirus diagnostic
assays, antibacterial peptide and transport peptide based on
15 peptides corresponding to the C-terminal domain of pestivirus
E^{rns} or the L3 loop of ribotoxin type II proteins. Preferred
regions used for peptide based diagnostics are listed in
table 2 and preferred membrane active peptides used for
antibiotics or transport peptide are listed in Table 4 or 5,
20 9 to 11. However, they can of course be used interchangeably
for their various uses. The length of the peptide to be used
in a diagnostic assay or vaccine is conveniently the exact
length of the domain (residues 191 to 227, 37 residues) but
can of course be shorter or longer, as long as it does not
25 essentially change in antigenic or immunogenic character. The
maximum length of a suitable peptide (residues 177 to 227, 51
residues) can incorporate a 14-residue linker region between
the RNase domain and the C-terminal domain. This linker
region may be exposed in case of uncertainty of the exact
30 spatial position of the C-terminal domain relative to the
RNase domain and because of the conformational change of the
C-terminal domain. For that reason, the linker region may be
part of a large C-terminal antigenic site. The preferred
minimum length of a suitable peptide to be used in a
35 diagnostic assay or vaccine is the part of the C-terminal
domain that forms a amphipathic helix. This is the part of

the C-terminal domain without the 5 C-terminal hydrophobic
residues (191 to 222, 32 residues).
The diagnostic assays based on the peptides can be used to
determine antibody levels in blood, serum, milk or other body
5   fluids.
The materials according to the invention can be used for
incorporation in vaccines, for example to provide for a
carrier of the desired antigen to antigen presenting cells,
or to present an antigen within the context of MHC (I or II)
10 peptide presentation, or to provide for mucosal vaccination
by providing translocation of a desired antigen over an
epithelial (gut) layer. The peptide can also be used to
transport various cargos in eukaryotic cells, as far as into
the Golgi system or in the nucleus of cells. Such cargoes can
15 comprise protein or peptide material, PNA, RNA or DNA, drugs,
secondary metabolites, and so on. Peptide mixtures could be
used as well, to provide for synergy in antibacterial
activity, transportation or translocation.

20 Examples.

Structure analysis of pestivirus E^{rns}
A detailed analysis of the primary structure and homology
modelling of pestivirus E^{rns} allowed the definition of
25 antigenic regions on the surface of the protein which can be
mimicked by single linear peptides. The C-terminal 37
residues (191 - 227) is the best candidate because of its
location on the outer rim on the surface of E^{rns} and because
it folds independently from the rest of the protein as a
30 subdomain and it is not masked by any potential carbohydrate.
The independent character of the C-terminus is also
illustrated by the functional analysis of E^{rns}. E^{rns} mutants
have been made which are truncated from residue 168. In these
mutants the whole C-terminal part from residues 169 to 227
35 is missing. This mutant is still able to fold natively
because the discontinuous active site is still intact and the

mutant has wildtype RNase activity Furthermore, the C-
terminal 37 residues don't align with the other RNases but
they do align with a L3 loop in ribotoxin type II proteins
and with membrane active peptides like magainins. These
5   membrane active peptides have a well-defined function and for
the magainins it has been shown that they adopt a helical
conformations if they contact the cell membrane. We have
demonstrated the membrane active property of the E^{rns} and or
L3. The membrane active properties of the E^{rns} peptide agrees
10   with the functionally independent nature of the subdomain.
The location of this subdomain, the possibly independent
folding of the sequence, the lack of potential glycosylation
sites and its biological function make a peptide representing
this region a suitable candidate to be used as
15   antigen/immunogen for immunoassays and vaccines. Furthermore,
the biological activity of the E^{rns} or the L3 peptide make
these peptides suitable candidates to be used as an
antibacterial agent and/or a transport peptide.

Elisa development

Peptide synthesis

Peptides were selected from the C-terminal region, (residues
5   191 - 227) of CSFV E^{rns}, strain Alfort 187, BVDV E^{rns}, strain
M96751 and BDV E^{rns}, strain X818 and the L3 loop of
restrictocin (residues 59-88; Lamy and Davies, NAR 19: 1001-
1006, 1991) and magainin (Zasloff, PNAS 84: 5449-5453, 1987)

10   CSFV : acetyl-ENARQGAARV TSWLGRQLRI AGKRLEGRSK TWFGAYA-COOH
CSFV : biotin-ENARQGAARV TSWLGRQLRI AGKRLEGRSK TWFGAYA-COOH
BVDV: acetyl-EGARQGTAKL TTWLGKQLGI LGKKLENKSK TWFGAYA-COOH
BVDV: biotin-EGARQGTAKL TTWLGKQLGI LGKKLENKSK TWFGAYA-COOH
BDV : biotin-ENARQGAAKL TSWLGKQLGI MGKKLEHKSK TWFGANA-COOH
15   restrictocin: biotin-GNGKLIKGRTPIKFGKADCDRPPKHSQNGMGK-NH_2
magainin: biotin-GIGKFLHSAGKFGKAFVGEIMKS-NH_2

Peptides were synthesized according to standard procedures on
an Applied Biosystems 430A synthesizer using Fastmoc
20   chemistry (Fields et al. Pept Res 4: 95-101, 1991). An extra
CSFV and BVDV peptide were synthesized which were N-
terminally biotinylated instead of acetylated.

Serum samples
25   The following swine serum samples were incorporated in the
study to evaluate the peptide Elisa's.

- Negative field serum samples (n=96) were randomly obtained
from slaughtered adult pigs. Sera were all tested negative in
30   the CSFV-E2 and pan-pestivirus antibody specific Ceditest
ELISAs.
- Pestivirus serum antibody-positive but CSFV-negative serum
samples (n=96) were randomly obtained from slaughtered adult
pigs. Swine sera were tested negative in Ceditest CSFV-E2
35   specific ELISA (Colijn et al.Vet Micro Biol. 59: 15-25, 1997)
and positive in the pan-pesti Elisa (Paton et al. J. Virol

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Meth. 31: 315-324, 1991; Kramps et al. Vet Micro Biol.64: 135-144, 1999).

-CSFV-antibody positive field serum samples tested by virus neutralisation test were obtained (n=95)from an infected pig
5   farm (VR) that was infected during the CSF epizootic in the Netherlands in 1997-1998.

- Sequential serum samples were collected during a vaccination/challenge experiment of 12 pigs that were vaccinated with E2 and infected with CSFV, strain Brescia.
10 Specific Pathogen Free (SPF) animals were challenged with the virulent CSFV strain Brescia, 2 weeks after a single vaccination with the E2 subunit vaccine.

- Panel of swine sera that were experimentally infected with BVDV (n=5, numbers 4 - 8).
15 - Panel of swine sera that were experimentally infected with CSFV strain Paderborn (n=5, numbers 9 - 13).

- Panel of bovine sera that were experimentally infected with BVDV (n=9, numbers 1 - 6, r4590-51, r4590-52, 841).

- Reference panel obtained from the European reference
20 laboratory for CSFV: sera from swine that were experimentally infected with CSFV (n=14), BVD (n=1) or BVDV (n=12). Three sera were obtained from swine with experimental mixed infections of BVDV/BDV (n=1) and CSFV/BVDV (n=2).

- Pool of hyperimmunesera against CSFV (HIS CSFV)
25 - Pool of hyperimmunesera against BVDV (HIS BVDV)

Solid phase peptide Elisa (sp-Elisa)

Test procedure

For the sp-Elisa a similar format was chosen as for a
30 previously developed RSV G-peptide Elisa (Langedijk et al.J.Imm. meth. 193: 153-166, 1996) One microgram of N-terminally acetylated pestivirus peptide was coated per well of a high binding capacity flat bottom microplate (Greiner) in 50 ul of carbonate buffer pH 9.0, 37°C and dried
35 overnight. The optimal dilution of the peptide to coat ELISA

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plates was chosen in such a manner that maximum binding was
obtained as determined in a checkerboard titration. Test sera
were titrated. Mouse-anti swine IgG (23.3.1b) conjugated to
horseradish peroxidase (HRP) was diluted 1:1000. Rabbit anti-
5 bovine IgG - HRP (P0159, Dako, Denmark) was diluted 1:1000.
Conjugates and test sera were incubated for 1h at 37°C in
ELISA buffer (8.1 mM Na_2HPO_4, 2.79 mM KH_2PO_4, 0.5 M NaCl, 2.68
mM KCl, 1 mM Na_2EDTA, 0.05% v/v Tween 80, pH 7.2) containing
4% horse serum. The substrate chromogen consisted of
10 ABTS/H2O2. Incubation was performed during 30 minutes at
22°C. OD was measured at 405 nm (Titertek multiscan).

Results

The reactivity of BVDV positive swine sera (4 - 8) and CSFV-
15 positive swine sera (9 - 13) were tested for reactivity in
the CSFV sp-ELSA and the BVDV sp-ELISA.
The reactivity of bovine sera (numbers 1 - 6, r4590-51,
r4590-52, 841) were tested for reactivity in the CSFV sp-
ELISA and the BVDV sp-ELISA.
20 Reactivity of the sera with the peptides was excellent which
shows that the peptides indeed correspond to an
immunodominant region of E^{ms}. This agrees with the prediction
of the immunodominant character of the subdomain. However,
the CSFV and BVDV sera are cross-reactive for both peptides.
25 Although the panel of CSFV-specific swine sera reacted better
than the panel of BVDV-specific swine sera in the CSFV ELISA
the reactivity of both panels of sera are similar in the BVDV
ELISA. Similarly, the panel of BVDV-specific bovine sera show
high reactivity in the BVDV peptide ELISA (Fig 4. d), but the
30 sera also cross react considerably in the CSFV ELISA (Fig. 4
c).

Liquid phase peptide Elisa (lp-Elisa).
Because of the high cross-reactivity in the solid phase
35 peptide Elisa, an Elisa was developed in which the antigen
was recognised in liquid phase (lp-Elisa). Moreover, by

labelling the homologous peptide of the pestivirus of
interest (CSFV peptide), unlabeled heterologous peptide of
the cross-reactive pestivirus (BVDV peptide) could be used to
block unspecific cross-reactivity.

5   In the liquid phase peptide Elisa for detection of
antibodies against CSFV, the test serum was incubated with a
mixture of biotinylated CSFV peptide and acetylated BVDV
peptide (without biotin). CSFV-specific antibodies will
preferably bind the biotinylated CSFV peptide and BVDV-
10 specific antibodies will preferably bind the non-biotinylated
BVDV peptide. Subsequently, the mixture is transferred to an
avidin-coated microtiterplate and the antibodies complexed to
the biotinylated CSFV peptide will be caught by avidin and
can be detected with an anti-swine peroxidase conjugate and
15 subsequent incubation with substrate.

Test procedure:

Avidin coated microtiter plates: 400 ng of ImmunoPure avidine
(No. 21121, Pierce, rockfort, Illinois, USA) in 100 ul of
20 carbonate buffer (pH 9) in each well of a high binding
capacity flat bottom microplate (Greiner). Plates were
covered and incubated overnight at 37°C. After coating the
plates were kept frozen until use.
Before use, the avidin-coated plates were incubated with 100
25 ul of phosphate buffered saline (PBS, pH 7) with 10 % horse
serum per well for 2 h at 37°C on a shaker.
Meanwhile, test serum (1:50) was incubated with a mixture of
10 ng biotinylated CSFV-peptide and 30 ng of BVDV-peptide in
100ul of Elisa buffer with 4 % of horseserum during 1 h, at
30 37°C.
Avidin-coated plates were washed and 100 ul of testserum and
peptides mixture was transferred in the wells and incubated
for 45' at 37°C. Subsequently, plates were washed and
incubated with 100 ul mouse-anti swine IgG (23.3.1b)
35 conjugated to horse radish peroxidase (HRP) (Van Zaane et al,
1987) diluted 1:1000 or with rabbit anti-bovine IgG - HRP

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(P0159, Dako, Denmark) diluted 1: The substrate chromogen
consisted of ABTS/H_2O_2. Incubation was performed during 30
minutes at 22°C. OD was measured at 405 nm (Titertek
multiscan). Cut off value was chosen at OD > 0.5 which is
5 approximately 3 times the average background of known
negative sera.

Results

The reactivity of BVDV positive swine sera (4 - 8) and CSFV-
10 positive swine sera (9 - 13) were tested for reactivity in
the CSFV lp-ELISA (Figure 5). This test format showed much
better specificity than the sp-peptide Elisa.

To determine the specificity of the lp-peptide-ELISA, 96
15 negative field serum samples were tested in the lp-peptide-
ELISA for CSFV E^{rns}-Ab. Only 2 of 96 samples showed a positive
response (cut off was chosen at OD > 0.5). Based on these
data, the specificity of the lp-peptide ELISA for CSFV E^{rns}-Ab
amounts to 98% (= 94/96 x 100%) (Figure 6).
20 To determine the specificity of the lp-peptide-ELISA, 96
field sera that contain antibodies directed against other
pesti-viruses than CSFV (BVDV and BDV) were tested in the lp-
peptide-ELISA. Only 2 of 96 samples showed a positive
response (OD > 0.5). Based on these data, the specificity of
25 the lp-peptide ELISA for CSFV E^{rns}-Ab, for non-CSFV-pestivirus
positive sera, amounts to 98% (= 94/96 x 100%) (Figure 6).

To determine the sensitivity of the lp-peptide-ELISA, 95
field serum samples from a CSFV-infected farm (VR) obtained
30 during the CSF epizootic in the Netherlands in 1997-1998 was
tested in the lp-peptide-ELISA. Not a single serum sample
showed a positive response (OD > 0.5). Based on these data,
the sensitivity of the lp-peptide ELISA for CSFV antibodies
amounts to 100% (Figure 6).
35 An interesting application of the lp-peptide ELISA is a
diagnostic test that can be used to detect CSFV infection of

29

E2 vaccinated pigs. Therefore, sera of E2 vaccinated pigs
should be unreactive in the lp-peptide ELISA and should be
positive after CSFV challenge of the pigs. Successive serum
samples, which were collected during a challenge experiment
5   of 12 E2-vaccinated pigs that were infected with CSFV, were
tested in the lp-peptide ELISA (Figure 7). The results show
that all sera but one from E2 vaccinated pigs were negative
prior to CSFV challenge. All animals (except 1) seroconverted
14 to 28 days after challenge. Finally, the performance of
10   the lp-peptide ELISA was compared with the E2-based Ceditest
ELISA and two other E^{rns}-based ELISAs. The reactivity of a
panel of European reference sera (see methods) was tested in
all four ELISAs (Table 6). Although the E2-based ELISA was
superior, (one false negative), the lp-peptide ELISA
15   performed better (three false negative) than the other ELISAs
based on epitope blocking using complete Erns (5 false-
negative, one false-positive and one false-negative, six
false-positive). It is very likely that the lp-peptide ELISA
can even be optimized when it is changed into an antibody
20   blocking format, like the other three ELISAs in Table 6.

To illustrate the compatibility of the peptide ELISA with
other pestiviruses, the CSFV-specific peptide ELISA was
changed into a BVDV and a BDV ELISA by exchanging the
biotinylated CSFV peptide for a biotinylated BVDV peptide or
25   a biotinylated BDV peptide, the acetylated BVDV peptide for
the acetylated CSFV. The same amount of peptide was used as
in the lp-CSFV peptide ELISA and all assay conditions were
kept similar. The panel of swine sera that were
experimentally infected with BVDV (n=5, numbers 4 - 8) or
30   CSFV (n=5, numbers 9 - 13) were tested in the three different
lp-peptide ELISAs for the three different pestivirus types.
Table 7 shows that BVDV-positive sera react best in the BVDV-
specific peptide ELISA and that the CSFV-positive sera react
best in the CSFV ELISA although the CSFV sera cross react to
35   some extent with the BVDV peptide. As expected on the basis
of the sequence homology, the BVDV and BDV ELISAs show less

differentiation. The BVDV and BDV ELISA both contained
acetylated CSFV peptide as competing antigen. Some
improvement may be possible when also acetylated BDV and BVDV
peptide would be used as competing antigen in the BVDV and
5   BDV ELISA, respectively.
The acetylated CSFV peptide (Table 2) was further used to
test the immunogenicity of the peptide in pigs and to examine,
whether vaccination with the peptide could protect the pigs
after CSFV challenge. Pigs were vaccinated with the various
10   amounts of the peptide, formulated in Freund's Complete
Adjuvant (FCA). Four weeks later the pigs were vaccinated
again with 1.3 mg peptide, formulated in Freund's Incomplete
Adjuvant (FIA). Five control pigs were vaccinated similarly
with just FCA and FIA. Three weeks after the second
15   vaccination the pigs were challenged nasally with 100 LD50
CSFV, strain Brescia 456610. Antibody reactivity against the
peptide was monitored during the experiment. Before and after
challenge virus isolations were performed on white blood
cells. After death or euthanasia, organs (tonsil, spleen,
20   kidney and ileum) were tested for the presence of viral
antigen using an immunofluorescence test.

Monoclonal antibody production

25   Production of E^{rns}-peptide specific monoclonal antibodies
(Mabs) was performed as described (Wensvoort et al., 1986).
Two BALB/c mice were immunized intraperitoneally with 400 ug
CSFV or BVDV peptide (residues 191 - 227), mixed with
Freund's complete adjuvant (FCA). After 4 weeks the mice were
30   boostered with 400 ug of peptide mixed in incomplete FCA and
3 weeks later the mice were boosterd with 400 ug of peptide
in phosphate buffered saline. Three days later the spleen
cells were fused with sp20 cells and hybridomas were grown in
selective medium. The Erns specificity of the produced Mabs
35   (Mab 906-2-1 (BVDV) and Mab 907-35-1-1 (CSFV))was determined

31

using an E^{rns} antigen detection ELISA. Additionally, the Mabs
reacted in the E^{rns} peptide-based ELISAs

5   Transport activity of E^{rns} peptide.
     Test procedure.
     Binding of E^{rns} peptide. Monolayers or cytospins of cell
     suspensions of several cell types (Ebtr, SK6, sf-21, Caco-2
     and HT-29) were fixed with acetone or 4%-paraformaldehyde.
10   Glass slides or coverslips with fixed cells were incubated
     with biotinylated CSFV peptide (100 or 10 ug/ml PBS) for 1h
     at 37°C. After washing with PBS, the slips were incubated
     with avidin-HRP (1:100, Zymed) or avidin-FITC (1:70, Zymed)
     for 30' at 37°C. Cells were inspected for specific binding of
15   the peptide by light microscopy (HRP) or fluorescence
     microscopy (FITC).

     Translocation of E^{rns} peptide. Translocation of the peptide
     across the plasma membrane was studied by incubation of live
20   cells in suspension or subconfluent monolayers on coverslips
     with biotinylated peptide (200 to 0.4 ug/ml culture medium)
     for 1, 10, 30, 45 or 180 minutes. After the time period,
     cells were fixed with 4% paraformaldehyde or cold methanol
     and labeled with avidin-FITC as described above. Fixed cells
25   were inspected with fluorescence microscopy. Internalization
     was established with confocal microscopy. The following cell
     lines were used : A72, canine fibroblast tumor cells; MDCK,
     canine kidney epithelial cells; CCO, sheat-fish ovaria cells;
     EK-1, Eel Kidney cells; CHS-E, salmon embryonal cells; BUEC,
30   Bovine umbillical endothelial cells; BFDL, Bovine feutal
     diploid lung cells (fibroblast); PUEC, Porcine umbillical
     endothelial cells; HT 29, colorectal adenocarcinoma, colon
     epithelial cells; CaCo-2, colorectal adenocarcinoma, colon
     epithelial cells; Hela, adenocarcinoma, cervix; Vero, normal
35   monkey kidney epithelial cells; SK6, Swine Kidney cells;
     NPTh, Newborn Pig Thyroid cells; ECTC, Embryonal Calf Thyorid

cells; MDBK, normal bovine kidney epithelial cells; EBTr,
Epithelial Bovine Trachea cells; Bovine sperm cells;
Sp20mouse myeloma B-cells.

5   *Trans epithelial transport.* The potential of E^{rns} peptide to
     permeabilize epithelium and assist transport of molecules
     across epithelium was tested in USSING chambers of the
     snapwell type with CaCo-2 or HT29 cells, that closely mimic
     epithelial cell sheets.
10   The potential of E^{rns} peptide to carry non-linked molecules
     across epithelium was tested by mixing HRP (0.08 ug/ml
     culture medium) with several concentrations of E^{rns} peptide
     (50, 5 and 0.5 ug/ml ringers medium) in the upper chamber.
     Samples were drawn from the lower chamber after 15, 30, 45,
15   60, 120 and 240 minutes, which were tested for HRP
     concentration.

     Transdermal transport. The potential of the an E^{rns} peptide to
     penetrate the skin was tested by applying 150 µl 0.3 - 4 mM
20   of biotinylated peptide in a chamber containing an isolated
     piece of `fresh' human breast skin or in a chamber which was
     glued on the skin of a live pig, or applying 50 µl of peptide
     solution on the skin with a cotton wool tip, or applying 50 µ
     l of a peptide solution, mixed with 50 µl of contact gel on
25   the skin for 30 minutes to 120 minutes. After the incubation
     time the pig was killed, the skin was cleaned, biopsied and
     a biopsy was frozen in liquid nitrogen. Cryosection of the
     skin samples were fixed on microscopic slides with aceton and
     incubated with streptavidin-FITC (1/100) for 30 minutes.
30

     Hemolytic assay. Hemolytic activity of various peptide
     concentrations were determined by incubation with human,
     guinee pig or sheep erythrocyte suspensions (final
     erythrocyte concentration, 1% v/v) for 1h at 37 C. After
35   cooling and centrifugation, the optical density of the

33

supernatants were measured at 540 nm. Peptide concentrations causing 50% hemolysis (EC_{50}) were derived from the dose-response curves.

5   *Clonogenicity of mammalian cells.* HeLa or EBTr cells were cultured in DMEM,
      supplemented with 20 % foetal bovine serum and antibiotics in a humidified atmosphere supplied with 5% CO2 at 37 °C. Exponentially growing cells were treated with trypsin and
10   transferred to wells of a 96-wells microtiter plate. Resulting in approximately 300 cells for each 30 μl of growth medium containing various oncentrations of peptide. After incubation for 75 min (the plates were incubated upside down to avoid anchorage), the cells were transferred and plated in
15   wells of tissue culture plates, which contained 100 μl of growth medium. Cell growth was checked after 3 to 6 days.

      Anti microbial assay. Two bacterial strains (Escherichia coli ATCC 25922 and Enterococcus faecalis ATCC 29212) were
20   inoculated on heart infusion agar with 5% sheep blood and incubated aerobically overnight at 37°C. From the pure cultures suspensions were made in saline to a density of 0.5 McFarland. These suspensions were tenfold diluted in Mueller Hinton II broth resulting in a final inocolum of
25   approximately 10^7 cfu/ml.
      Standard 96 wells micro titre trays were filled with 100 μl of twofold dilutions of peptide in physiologic salt solution in each well resulting in the following concentration range: 4000, 2000, 1000, 500, 250, 125, 62.25, 31.63, 15.82, 7.96
30   and 0 μg/ml. The in column 12 were filled with 200 μl MH II broth (negative control).
      Columns 1 - 11 of the micro titre trays were filled with 100 μl of the final inoculum of the bacterial suspensions, thus diluting the concentrations of the peptide twofold resulting
35   in the following peptide concentration range in the wells:

34

2000, 1000, 500, 250, 125, 62.25, 31.63, 15.82, 7.96, 3,98
and 0 μg/ml. In rows B, C and D 100 μl of the final inoculum
of *E. coli* and rows E, F and G 100 μl of the final inoculum
of *E. faecalis* was pipetted. The final bacterial

5   concentration was approximately 5 x 10^5 cfu/well.
All trays were sealed and incubated overnight at 37°C.
After incubation the micro titre trays were inspected
visually for bacterial growth and the absorbance of the
cultures at 630 nm (A_{630}) was determined with an Elisa-reader.

10   The peptide concentrations in the wells with the lowest
concentrations that showed no visible growth or increase in
absorbance compared with the negative control wells, were
considered to be the Minimum Inhibitory Concentrations
(MICs). The experiment was performed independently in

15   triplicate. The test was repeated again in triplicate after
two days with the final peptide concentration range in the
wells: 5000, 2500, 1250, 625, 312.5, 156.25, 78.12, 39.06,
19.58, 9.79, 4.88, 2.44 and 1.22 μg/ml.

20   *Peptide synthesis.* A panel of truncated E^{rns} peptides was
synthesised to elucidate the minimal membrane active region.
A panel of truncated restrictocin L3 peptides was synthesised
to elucidate the minimal membrane active region.
Substitutions in the amino acid sequence of transport peptide

25   module can be applied to increase the translocation activity.
An optimized transport peptide can for example be synthesized
according to retro-inverso peptide chemistry, in which the
sequence is reversed and D-amino acids are used instead of L-
amino acids. Synthesis is performed as described above.

30   *Coupling of peptide to oligonucleotide.* The optimized E^{rns}
peptide containing a Broom-acetic acid at it's N-terminus
(Broom-GRQLRIAGKRLEGRSK) coupled to two sulfhydryl groups at
the 5'- and the 3'-end of a FITC-labeled 32 residues long
oligonucleotide (Thiol-GT^{FITC}CCACCGAGGCTAGCTACAACGACCCTTATAT-

35   thiol).

35

<u>Results</u>

To determine the binding of the E^{rns} peptide, biotinylated
5   CSFV peptide were incubated with various fixed cells. Binding
was determined after incubation with avidin-HRP or avidin-
FITC. Biotinylated CSFV peptide was able to bind to all
tested cell types. There was a marked difference between the
binding to paraformaldehyde versus acetone fixed cells. In
10 contrast to the paraformaldehyde-fixed cells, the acetone-
fixed cells showed less binding with the peptide. Because
much of the membrane fraction gets washed away after acetone-
fixation, this suggests that the peptide may bind the
membrane.
15 Next, cell suspensions (mouse myeloma and bovine sperm) and
subconfluent monolayers of various cell types (see test
procedure) were incubated with biotinylated CSFV peptide and
fixed after different time intervals. Inspection with
fluorescent microscopy and confocal microscopy showed that
20 the peptide had penetrated inside all cell types. The peptide
entered the cell within 1 minute, optimal fluorescence was
established after 30 minutes (Figure 8 and 9). Peptide was
translocated to specific regions inside the nucleus, which
may be the nucleoli. Peptide was also distributed around the
25 nucleus in membranous parts in the cytosol. The
colocalisation in the nucleoli was established by double
staining with acridinorange (Merck, Darmstadt, Germany), and
streptavidin - texas red. Yellow Fluorescence was observed
which indicates co-localisation of peptide and nucleoli
30 stain. Next, the membrane active region of the peptide was
precisely defined by testing the translocation activity of a
panel of truncations of the E^{rns} peptide and some peptides
with N-terminal additions and C-terminal deletions (Table 8).
Translocation to the nucleus was more effective with the
35 CSFV, strain Alfort, E^{rns} peptide compared to the BVDV or BDV
E^{rns} peptide. The CSFV, strain Alfort, E^{rns} peptide

36

translocated also more effective than the CSFV peptide
corresponding to strains in which positions 209, 210 and 217
were substituted. Furthermore, deletion of the seven most C-
terminal residues and the three N-terminal residues increased
5   the nuclear translocation activity of the peptide. To reduce
the length of the transport peptide further, the region
responsible for translocation (residues 194 -220) was
truncated while arginines were introduced to maintain, or
enhance the translocation activity. N-terminal deletions were
10 made of 4 and 5 residues and one extra arginine was
introduced at the same face of the presumed helix. These
shorter peptides retained translocation activity (Table 9).
Next, 11 N-terminal residues were deleted and the glutamic
acid was substituted by arginine. This susbstitution enhanced
15 the translocation activity by a factor of 33. Replacing the
two lysine residues by arginine residues enhanced the
translocation activity by an additional factor 3. Introducing
more arginines in the sequence did not improve the
translocation activity of the biotinylated peptide (Table 9).
20 Next, the optimised peptide (MDK-20) was synthesized with D-
amino acids according to the retro-inverso approach with an
additional lysine-MTT (peptide A941: biotin-
rsrgrlrrgairlqrgK(MTT)-BrHAc). This peptide retained its
translocation activity and showed even a higher translocation
25 activity compared with the original MDK-20, showing the
advantages of the retro-inverso approach.
Because of the homology between the E^{rns} peptide, magainin and
the L3 loop peptide, the biotinylated magainin and L3 loop
peptide were also tested for translocation activity. L3
30 showed translocation activity, magainin did not (Figure 9).
Next, the membrane active region of the peptide responsible
for translocation was precisely defined by testing the
translocation activity of a panel of truncations of the L3
peptide (Table 10). To elucidate general rules for peptide
35 translocation, we searched for new transport peptides and
tested them for translocation across the plasma membrane.

HIV-1 tat peptide which is a transcription factor that binds
DNA, the AntP peptide which is a homeobox protein and
transportan which is a hybrid peptide of a bee venom and
galparan. The only similarity between the known transport
5   peptides is the content of basic residues. See also Wender,
P. A. et al., 2000. Proc Natl Acad Sci U S A. 97:13003-8,
who, however, require high >50% arginine content.
To search for more transport peptides, we tested the
translocation of basic peptides which were derived from
10   linear DNA-binding motifs; RNA-binding motifs; heparin
binding motifs, basic enzymatic cleavage sites and nuclear
localization signals. For nuclear localization signals (NLS)
it is already known that they are able to translocate the
nuclear membrane, although it is thought that they need to be
15   recognized by a receptor in order to translocate. We tested
whether peptides corresponding to nuclear localization sites
could also be used as general transport peptides that cross
the plasma membrane.

20   Several types of short transport peptides were found:

Monopartite: PKKKRKV. Binds the import receptor *Imp
alpha/imp beta* complex.

25   This sequence is identical to the NLS of simian virus 40
large T antigen.

Bipartite: KRPAAIKKAGQAKKKK (lysine rich signals).
30

NLS of HIV-1 Rev   RQARRNRRRRWR

HIV-1 rev is a viral RNA export factor and shuttles RNA
outside the nucleus. The sequence motif binds RNA.
35

38

A synthetic peptide has been developed (RSG-1.2)   that binds the Rev binding site RNA with 10 times higher affinity

DRRRRGSRPSGAERRRRR

5

NLS of human herpesvirus-8 K8 protein   TRRSKRRSHRKF

K8 protein is homologous to EB1 protein of Epstein-Barr virus (EBV) which is an transcriptional activator which directly
10   binds specific sites on the DNA.

Another class of basic peptides that may in some cases also be NLS peptides are RNA / DNA binding peptides. For instance the HIV-1 Rev peptide also binds directly to RNA.

15

Other examples of peptides that bind DNA or RNA:

RNA binding element of   flockhouse virus (FHV)
        NRTRRNRRRVR

20

RNA binding element of   bacteriophage lambda-N
        QTRRRERRAEKQAQW

Another class of basic peptides are basic enzymatic cleavage
25   sites

An example of a viral enzymatic cleavage site is in the surface proteins of alpha viruses. The cleavage site between E3 and E2 is highly basic. After cleavage it is the C-
30   terminal region of E3 which is located at the distal end of the viral spike, interacting primarily with E2.

Western equine encephalitis virus   E3: KCPSRRPKR.

35   Another example of basic peptides are linear heparin binding sites. The transport peptide corresponding to the E^{rns} C-

terminal domain also binds heparin. Examples are peptides
found in HRSV-G, type A:
KRIPNKKPGKKTTTKPTKKPTIKTTKKDLKPQTTKPK
and HRSV-G,   type B: KSICKTIPSNKPKKK

5   These peptides showed translocation activity (Table 11). In
the case of the heparin binding sites of HRSV-G, the region
responsible for translocation was mapped (Table 11). One of
the most active HRSV-G peptides (Biotin-KRIPNKKPKK) was
further optimized by changing all lysine residues into
10   arginine residues to check whether the peptide showed higher
translocation activity when residues were introduced which
were more basic. Changing the four lysines to argines
improved the translocation activity by a factor of 3 (Table
11).

15

According to Table 9, the most active biotinylated peptide
(residues 194 - 220) was still (faintly) detectable at 125
nM. As a result, the elucidation of the minimal essential
part of the translocation peptide also proves that a C-
20   terminal cargo of at least 6 residues can be transported and
a N-terminal cargo of at least 13 residues plus a biotin can
be transported. Because the peptide is probably also able to
transport the RNase domain of E^{rns} inside the cell, it is also
expected that large proteins can be transported by the
25   peptide. After mixing equimolar amounts of streptavidin-FITC
(~~52~~ 60 kD, nonglycosylated, neutral) with the most active E^{rns}
peptide (residues 194-220), and with an optimized E^{rns} peptide
(biotin-GRQLRIAGKRLEGRSK) it was possible to transport
streptavidin-FITC inside the cell and the nucleus. (Figure
30   10) The peptides were also able to carry avidin-TexasRed (66
kD, glycosylated, positively charged) inside the cells. Also
the optimised E^{rns} peptides and the long and short HRSV-G type
A peptides (MDN-12 and MDP-32 according to Table 11) were
tested for their ability to transport streptavidin-FITC into
35   the cell (f - h). The translocation activity of the peptide
complexed to the streptavidin cargo was different from the

translocation of the biotinylated peptide alone. The
optimised E^{rns} peptide MDM-27, which has two additional
positive charges compared to the optimised E^{rns} peptide MDK-
20, transports streptavidin-FITC much better than MDK-20. In
contrast, in the case of the uncomplexed biotinylated peptide
the translocation activity was similar. This effect was even
more pronounced when the longer HRSV-G peptide (MDN-12) was
compared to the shorter HRSV-G peptide (MDP-32), which has
less positive charge but higher translocation activity as a
single, uncomplexed molecule without cargo (Figure 10 g,h).
To check whether the transport peptide was also able to
transport proteins that retain biological activity, the
peptide was complexed with streptavidin conjugated to the
enzyme ß-galactosidase (600 kD). The complex was transported
efficiently into the cells and retained it's ability to
release non-reducing terminal galactose. To check whether the
peptides could transport oligonucleotides, the optimized E^{rns}
peptide which was activated by a Broomacetic acid. Transport
modu;e Broom- GRQLRIAGRRLRGRSR was coupled to a FITC-labeled
32 nucleotide oligo at the 5'- and the 3'-end. The complex
was tested for translocation inside the cell and the nucleus.
Titration of the uncoupled oligonucleotide and the complex of
peptide and oligo showed that the intracellular accumulation
of the oligonucleotide was 75 times higher when it was
coupled to the transport peptide. To check whether the
membrane destabilising activity had a general toxic effect on
cells, leakage of the cell was tested for tryphan blue after
peptide incubation of 30 minutes. Only at high concentrations
of peptide (>35 uM) some tryphan blue could be determined
inside the cell, especially in isolated areas in the nucleus.

Hemolysis of erythrocytes can also be indicative for lytic
effect of the peptides on eukaryotic cell membranes.
Hemolysis of erythrocytes from several species was tested
with the panel of E rns peptides (Table 8). The different
peptides show a broad range of hemolytic activities on guinee

pig erythrocytes. The peptide with the highest translocation
activity (residues 194 - 220) has a low hemolytic activity.
No significant hemolysis was observed with sheep and human
erythrocytes. The effect of the E rns peptide on cell growth
5   of HeLa cells and EBTr cells was determined in a
clonogenicity assay as shown in Table 12. These data
correspond to the other toxicity assays and indicate that the
translocation activity is much higher than the cytotoxic
activity.
10

Transdermal transport.
Next it was tested whether the optimized E^{rns} peptide (MDK-20)
was able to penetrate an epithelial layer. Because of the
stratum corneum layer, the skin seems to be the most
15   difficult epithelial barrier to take. To test the penetration
by the biotinylated peptide, a sample of human breast skin
was contacted with the biotinylated peptide in vitro during 2
hours. In fixed cryosections of the skin, the biotinylated
peptide could be visualized with streptavidin-FITC in the
20   epidermis and the dermis (Figure 13). The same results were
obtained when the peptide was contacted with the skin of a
pig in vivo. As soon as 30 minutes after application,
penetration of the peptide into the epidermis was observed.
Because of the high amount of proteolytic enzymes in the
25   skin, transdermal transport was also tested with the stable
retro-inverso peptide containing D-amino acids. Much more
accumulation in the skin was observed for the peptide with D-
amino acids (A941) compared with the original peptide
containing L-amino acids (MDK-20)
30

Trans epithelial transport. Next, the peptide was tested for
the ability to assist leakage of proteins through an
epithelial cell sheet. HorseRadish Peroxidase which was mixed
35   with the peptide could be transferred through the cell sheet.

Antibacterial activity. Because of the membrane activity and
the homology with antibacterial peptides, the antibacterial
activities of the peptides were determined as described
5   above. The peptides indeed showed antibacterial activity
against the gram-negative *E. coli* (Table 13) but not against
E. faecalis. The MICs against *E. coli* correlate with the
translocation activity of the peptide. The restrictocin L3
peptide showed no antibacterial activity.

10

Descriptions of the figures

Figure 1.

5   Schematic representation of alignment of pestivirus E^{rns} with RNase Rh which indicates the modular organisation of E^{rns}. E^{rns} consists of an RNase domain (dotted) and a C-terminal membrane active domain (filled black). The C-terminal domain (residues 191 - 227) which shows resemblance to the L3 loop

10   of cytotoxic RNases is described in this invention. RNase active site domains are shown as chequered boxes. Potential glycosylation sites are shown as ellipses.

Figure 2.

15   Helical wheel representation of residues 194 - 220 of CSFV E^{rns}.

Figure 3.

Sequence alignment of pestivirus E^{rns} C-terminal domains with

20   magainin and the L3 loop of restrictocin. Residues within one distance unit from magainin are boxed. Units are defined in structural distance table in the magalign package of DNASTAR, Inc. The Structural table scores for residues that are chemically and spatially similar. All identities score a

25   value of 6. Mismatches score less than identities. The Structural table is designed for use with the Jotun-Hein method.

30   Figure 4.

Crystal structure of RNAse Rh.

Figure 5.

35   a. Reactivity (optical density, OD) in CSFV lp-peptide Elisa of dilutions of BVDV specific swine sera (4b - 9b) and CSFV

44

specific swine sera (9c to 13c) and CSFV-specific hyperimmune
serum.

b. Reactivity in CSFV lp-peptide Elisa of dilutions of BVDV
specific bovine sera (1 - 6, r4590-51, r4590-52, 841) and
5   BVDV-specific hyperimmune serum.

Figure 6.
Reactivity of several panels of sera in the CSFV lp-peptide
ELISA as described in the test procedure.
10   Negative field serum samples (n=96) were randomly obtained
from slaughtered adult pigs and were all tested negative in
standard pestivirus ELISA. Pestivirus positive but CSFV-
negative serum samples (n=96) were randomly obtained from
slaughtered adult pigs. CSFV-positive field serum samples
15   were obtained (n=95) from an infected farm (VR) that was
infected during the CSF epizootic in the Netherlands in 1997-
1998.

Figure 7.
20   Reactivity of successive serum samples collected during a
vaccination/challenge experiment in the CSFV lp-peptide
Elisa. Twelve pigs were vaccinated with E2, 14 days before
challenge.

25   Figure 8.
Distribution of biotinylated CSFV E rns peptide (a,c) and
biotinylated control peptide (b) (25 µM) after 30 minutes of
incubation with subconfluent EBTr cells grown on a 10-well
microscope slide. Cells were fixed with cold methanol and
30   biotinylated peptide was visualized by staining with avidin-
FITC for 30 minutes. Fluorescent micrograph (250 X)(a,b) or
fluorescent micrograph using confocal microscope (600 X) (c).

Figure 9.
35   Distribution of biotinylated L3 peptide (a) and magainin-1
peptide (b) (6 µM) after 30 minutes of incubation with

subconfluent EBTr cells grown on a 10-well microscope slide.
Cells were fixed with cold methanol and biotinylated peptide
was visualized by staining with avidin-FITC for 30 minutes.
Fluorescent micrograph (250 X).

Figure 10.
Transport of avidin and streptavidin. Equimolar amounts of
avidin-TexasRed (6̶8̶ 66 kD) (a) or streptavidin-FITC (5̶2̶ 60
kD) (c) were mixed with the biotinylated Erns peptide or
unbiotinylated peptide (b,d) (residues 194-220) (3 µM) and
incubated with EBTr cells for 30 minutes.
Transport of a complex of streptavidin-FITC with optimized
E^{rns} peptide biotin-GRQLRIAGRRLRGRSR (e), optimized E^{rns}
pepitde biotin-GRQLRRAGRRLRRRSR (f), HRSV-G type A peptide
biotin-KRIPNKKPGKKTTTPTKKPTIKTTKKDLKPQTTKPK (g) and HRSV-G
type A biotin-KRIPNKKPGKKT (h).

Figure 11.
Distribution of FITC-labled oligonucleotide (32nt) (a) and
FITC-labeled oligo coupled to optimised E^{rns} peptide (b) at 56
µg oligo/ml after 30 minutes incubation with cells.

Figure 12.
Passage of HRP through epithelial cell sheet incubated with
0, 0.5, 5 and 50 µg/ml BVDV E^{rns} peptide.

Table 5

Restrictocin	GNGKLIKGRTPIKFGKADCDRPPKHSQNGMGK
Mitogillin	--------------------------------
Toxin AspfI	--------------------------------
Alpha-sarcin	-D---P----------S---------KD-N--
Gigantin	-E--IL----------S---------KD-N--
Clavin	-D--IL-------W-NS---------K--D--

Table 6. Comparison of reactivity of reference sera with different CSFV diagnostic tests

No	DPI[1]	Inoculum	E2-ELISA Cedi-E2[2]	E^RNS ELISA		
				Cedi-Erns[3]	Bommelie[4]	Peptide
1	43	CSFV Visbek/Han 95	+	+	+	0,171
2	16	CSFV Visbek/Han 95	+	+	+	0,477
3	20	CSFV Visbek/Han 95	+	+	+	1,796
4	20	CSFV Visbek/Han 95	+	-	+	3,165
5	14	CSFV Visbek/Han 95	+	+	+	0,7
6	21	CSFV Alfort 187	+	-	-	0,186
7	29	CSFV Diepholzl/Han 94	+	+	+	2,25
8	29	CSFV Diepholzl/Han 94	+	-	+	2,619
9	29	CSFV Diepholzl/Han 94	+	-	+	1,857
10	34	CSFV Visbek/Han95	+	-	+	3,87
11	55	CSFV Visbek/Han95	+	+	+	1,122
12	93	CSFV C-strain	+	+	+	0,543

No.	Days	Virus				Value
13	69	CSFV Diepholz1/Han 94	+	+	+	2,146
14	28	CSFV Diepholz1/Han 94	-	+	+	1,103
15		BVDV NADL	-	-	-	0,103
16		BDV	-	-	-	0,45
17		BVDV 2214	-	+	+	0,161
18		BVDV NADL	-	-	-	0,118
19		BVDV NADL + BDV	-	-	+	0,136
20		BVDV + CSFV Alfort 187	+	+	+	3,208
21		BVDV Osloss	-	-	-	0,146
22		BVDV Osloss	-	-	-	0,151
23		BVDV Osloss	-	-	-	0,151
24		BVDV Osloss	-	-	-	0,162
25		BVDV Osloss	-	-	-	0,172
26		BVDV Osloss	-	-	+	0,164
27		BVDV Osloss	-	-	+	0,149
28		BVDV Osloss	-	-	+	0,163
29		BVDV Osloss	-	-	+	0,215
30		CSFV Alfort 187+ BVDV Osloss	+	+	+	0,567

[1] Days post infection. Serum numbers 21 - 29 obtained from same animal

[2] Cedi-E2 assay registration no.: BFAV/KSP/D10/98

[3] Cedi-Erns assay

[4]Bommeli AG/ Intervet assay

Table 7. Comparison of reactivity (OD 405) in different
Pestivirus peptide ELISAs

Serum number	CSFV	BVDV	BDV
4 BVDV	0.236	1.346	0.781
5 BVDV	0.129	0.724	0.388
6 BVDV	0.106	3.211	0.824
7 BVDV	0.250	4.000	4.000
8 BVDV	0.104	4.000	2.325
9 CSFV	0.487	0.367	0.349
10 CSFV	2.090	1.612	0.343
11 CSFV	2.555	0.610	0.246
12 CSFV	1.133	0.453	0.412
13 CSFV	1.253	0.573	0.436
HIS CSFV 1:500	2.263	0.471	0.624
HIS CSFV 1:1000	1.419	0.267	0.356
HIS CSFV 1:2000	0.871	0.166	0.180
Negative serum	0.152	0.150	0.153

5   All sera were diluted 1:50 except for the HIS sera

Table 8. Determination of minimal membrane active sequence of E^{rns}.

Residue number	Sequence[1]	Cellular fluorescence	Nuclear fluorescence	Hemolysis (mg/mL)
191-227	$ENARQGAARVTSWLGRQLRIAGKRLEGRSKTWFGAYA#-COOH	++++	++	0.07
191-227[2]	$ENARQGAARVTSWLGRQLSTAGKRLE*RSKTWFGAYA#-COOH	+++	+	0.08
191-227 (BDV)	$ENARQGAAKLTSWLGKQLGIMGKKLEHHSKTWFGANA-COOH	++++	+	0.25

194-230[3]	$EGARQGTAKLTTWLGKQLGTAGKKLENKSKTWFGAYA#	++++	+	0.25
184-223	$DGMTNTIENARQGAARVTSWLGRQLRIAGKRLEGRSKTWF#	++++	++	0.15
181-220	$YLLDGMTNTIENARQGAARVTSWLGRQLRIAGKRLEGRSK#	++++	+++	0.09
177-216	$DTALYLLDGMTNTIENARQGAARVTSWLGRQLRIAGKRLE#	++	-	0.23
172-211	$GSLLQDTALYLLDGMTNTIENARQGAARVTSWLGRQLR	+	-	>0.33

	IA#			
191-223	$ENARQGAARVTSWLGRQLRIAGKRLEGRSKTWF#	+++	+++	0.15
191-220	$ENARQGAARVTSWLGRQLRIAGKRLEGRSK#	+++	+++	0.31
191-216	$ENARQGAARVTSWLGRQLRIAGKRLE#	++	+	>0.33
191-211	$ENARQGAARVTSWLGRQLRIA#	+	-	>0.33
194-220	$RQGAARVTSWLGRQLRIAGKRLEGRSK#	++++	++++	0.26
196-220	$GAARVTSWLGRQLRIAGKRLEGRSK#	+++	++	0.16
199-220		++	+	0.18

	$RVTSWLGRQLRIAGKRLEGRSK#	+	+	.
202-220	$SWLGRQLRIAGKRLEGRSK#	+	+	>0.33
205-220	$GRQLRIAGKRLEGRSK#	+	-	>0.33
MAGAININ	$GIGKFLHSAGKFGKAFVGEIMKS#	-	-	?
L3 loop 59-88	$GNGKLIKGRTPIKFGKADCDRPPKHSQNGMGK#	++++	++++	?

[1] $ = biotin, # = amide, * = mixture of G and R at that position of the peptide, bold italic residues differ from E[rns] of CSFV, strain Alfort.

[2] sequence corresponds to distinct CSFV strains present in sequence database.

[3] sequence corresponds BVDV, strain M96751.

[4] sequence of peptide which showed highest activity.

Table 9. Optimization of Erns peptide

peptide	Code	Sequence	Concentration (µM)
Translocation domain E^{rms} according to Table8	MDB-17	Biotin-RQGAARVTSWLGRQLRIAGKRLEGRSK-NH2	1,0
	MDL-8	Biotin-RRVTSWLGRQLRIAGKRLEGRSK-NH2	1,0
	MDL-9	Biotin-RVRSWLGRQLRIAGKRLEGRSK-NH2	1,0
	MDA-19	Biotin-GRQLRIAGKRLEGRSK-NH2	10
	MDR-25	Biotin-GRQLRIAGKRLRGRSK-NH2	0,3
Optimized transport peptide (movin)	MDK-20	Biotin-GRQLRIAGRRLRGRSR-NH2	0,1
Rhodamine labeled	A931	Rhodamine-GRQLRIAGRRLRGRSR-NH2	3,0
Mutations in movin	MDM-25	Biotin-GRQLRRAGRRLRGRSR-NH2	0,1
	MDM-	Biotin-GRQLRIAGRRLRRRSR-NH2	0,1

	26	Biotin-GRQLRRAGRRLRRRSR-NH2	0,1
Shorter version of movin	MDM-27	Biotin-RQLRIAGRRLRGRSR-NH2	0,1
Movin + bromoacetic acid biotine with D-aminozuren (retro-inverso)	MDM-28 A941	Biotine-RSRGRLRRGAIRLQRG-Lysine (MTT)-broomacetic acid	0,03

Table 10. Mapping of restrictocin L3 loop

Peptide	Sequence	Concentration (µM)
Restrictocin L3	Biotin-GNGKLIKGRTPIKFGKADCDRPPKHSQNGMGK-NH2	1,0
	Biotin-GNGKLIKGRTPIKFGKADCDRPPKHSQNGM-NH2	3,0
	Biotin-KLIKGRTPIKFGKADCDRPPKHSQNGMGK-NH2	0,3
	Biotin-KLIKGRTPIKFGKADCDRPPKHSQNGK-NH2	0,3
	Biotin-KGRTPIKFGKADCDRPPKHSQNGMGK-NH2	3,0
	Biotin-KLIKGRTPIKFGKADCDRPPKHSGK-NH2	0,3
	Biotin-KLIKGRTPIKFGKARCRRPPKHSGK-NH2	0,3
	Biotin-KLIKGRTPIKFGK-NH2	

5

Table 11. Translocation activity of transport peptides

Other heparin binding peptides

Name	Code	Sequence	Concentration (µM)
HRSV-G, type A	MDN-12	Biotin-KRIPNKKPGKKTTKPTKKPTIKTTKKDLKPQTTKPK-NH2	1,0
	MDN-13	Biotin-KRIPNKKPGKKTTTKPTKKPTIKTTKKDLK- NH2	1,0
	MDP-04	Biotin-KRIPNKKPGKKTTTKPTKKPTIKTTKK-NH2	0,3
	MDP-08	Biotin-KRIPNKKPGKKTTTKPTKKPTIK-NH2	0,3
	MDP-19	Biotin-KRIPNKKPGKKTTTKPTKK-NH2	0,3
	MDP-32	Biotin-KRIPNKKPGKKT-NH2	0.03
	MDS-34	Biotin-KRIPNKKPGKK-NH2	0.03
	MDS-	Biotin-KRIPNKKPKK	0.03

	36		
	MDS-09	Biotin-KKPGKKTTTKPTKKPTIKTTKK-NH2	0,3
	MDS-23	Biotin-KKPGKKTTTKPTKK-NH2	0,3
		Biotin-KKTTTKPTKK-NH2	
	MDS-37	Biotin-KKPTIKTTKK-NH2	0,3
HRSV-G, type B, region 1	MDP-21	Biotin-KSICKTIPSNKPKKK-NH2	1,0
	MDS-35	Biotin-KTIPSNKPKKK-NH2	0,1
HRSV-G, type B, region 2		Biotin- KPRSKNPPKKPK	

NLS and DNA/RNA binding peptides

Name	Sequence	Concentration (μM)
HIV-1 Rev	Biotin-DTRQARRNRRRRWRERQRAAAAR-NH2	0,1
	Biotin-RQARRNRRRRWR-NH2	0,3 / 0,1
RSG-1.2	Biotin-DRRRRGSRPSGAERRRRRAAAA-NH2	0,1
	Biotin-RRRRGSRPSGAERRRRR-NH2	1,0 / 0,3
HIV-1 tat/tar	Biotin-RPRGTRGKGRRIRR-NH2	0,3
Bacteriophage lambda N peptide	Biotin-QTRRRERRAEKQAQW-NH2	1,0
	Biotin-RRRERRAEK-NH2	1,0
Flockhouse virus peptide	Biotin-NRTRRNRRRVR-NH2	0,03
Monopartite, NLS simian virus 40 large T antigen	Biotin-PKKKRKV-NH2	0,1
Bipartite	Biotin-KRPAAIKKAGQAKKKK-NH2	0,1
Herpesvirus 8 K8 protein (res 124-135)	Biotin-TRRSKRSHRKP-NH2	0,1

Proteolytic cleavage site of viral surface protein

Alpha virus E3	Biotin-KCPSRRPKR-NH2	3,0

Antibacterial peptide

Antibacterial peptide Buforin	Biotin-TRAGLQFPVGRVHRLLRK-NH2	3,0

Table 12. *Peptide concentration that inhibits cell growth*

Cell type	E rns peptide (191 - 227) (μM)	E rns peptide (194 - 220) (μM)
HeLa	50	40
EBTr	50	60

Table 13. Antibacterial effect of E^{rns} or L3 peptides

Peptide[1]	MIC (ug/ml)[2]
191-227	47
191-227	91
194-230	93
184-223	46
181-220	51
177-216	224
172-211	>500
191-223	41
191-220	47
191-216	95
191-211	395
194-220	50
194-218	25
196-220	25
199-220	23
202-220	179
205-220	>500
L3	>500

1 Same peptides as in Table 8.

5   2 Minimal inhibitory concentration needed to inhibit bacterial growth.

Claims

1.   An isolated, synthetic or recombinant peptide module or
functional equivalent thereof comprising an amino acid
sequence that is at least 50% identical to an amino acid
5   sequence of a peptide capable of binding heparine.

2.   A module or functional equivalent thereof according to
claim 1 comprising an amino acid sequence that is at least
50% identical to an amino acid sequence of a peptide located
from at about amino acid position 194 to 220 in a pestiviral
10   E^{rns} protein RNase or located at from at about amino acid
position 59 to 88 in a L3 loop of a cytotoxic Rnase of a
ribosome-inactivating protein or located from at about amino
acid position 187 to 223 in a respiratory syncytial virus G-
protein.

15   3.   An isolated, synthetic or recombinant protein module or
functional equivalent thereof comprising an amino acid
sequence that is at least 50% identical to an amino acid
sequence of a peptide located from at about amino acid
position 194 to 220 in a pestiviral E^{rns} protein RNase or
20   located at from at about amino acid position 59 to 88 in a L3
loop of a cytotoxic Rnase of a ribosome-inactivating protein
or located from at about amino acid position 187 to 223 in a
respiratory syncytial virus G-protein.

4.   A module according to claim 1, 2 or 3 that is at least
25   70% identical to said amino acid sequence.

5.   A module according to claim 4 that is at least 85%
identical to said amino acid sequence.

6.   A module according to anyone of claims 1 to 5 wherein
said peptide comprises
30   RQGAARVTSW LGRQLRIAGK RLEGRSK or
RQGTAKLTTW LGKQLGILGK KLENKSK or
RVGTAKLTTW LGKQLGILGK KLENKTK or

RQGAAKLTSW LGKQLGIMGK KLEHKSK or

GNGKLIKGRTPIKFGKADCDRPPKHSQNGMGK or

GDGKLIPGRTPIKFGKSDCDRPPKHSKDGNGK or

GEGKILKGRTPIKFGKSDCDRPPKHSKDGNGK or

5   GDGKILKGRTPIKWGNSDCDRPPKHSKNGDGK or

KRIPNKKPGKK or

KTIPSNKPKKK or

KPRSKNPPKKPK or

a functional part thereof.

10   7.   A peptide module or functional part thereof wherein at
least said functional part of said peptide comprises a
reversed amino acid sequence to one of a sequence given in
claim 1 to 6 and wherein D-amino acids are used instead of L-
amino acids.

15   8.   A compound provided with a module or functional part
thereof according to anyone of claims 1 to 7.

9.   A compound according to claim 9 provided with a
targeting means.

10.   A pharmaceutical composition comprising a module

20   according to anyone of claims 1 to 7 or of a compound
according to claim 8 or 9.

11.   Use of a module according to anyone of claims 1 to 7 or
of a compound according to claim 8 or 9 for the preparation
of a composition such as a pharmaceutical or cosmetic

25   composition capable of membrane translocation.

12.   Use of a module according to anyone of claims 1 to 7 or
of a substance according to claim 8 or 9 for the preparation
of a composition such as a pharmaceutical or cosmetic
composition capable of eliciting antibiotic activity.

30   13.   A method for translocating a compound over a membrane of
a cell comprising providing said compound with a module
according to anyone of claims 1 to 7 and contacting it with a
cell.

14.   A method according to claim 14 wherein said compound

35   comprises a molecular weight of up to 600 kD.

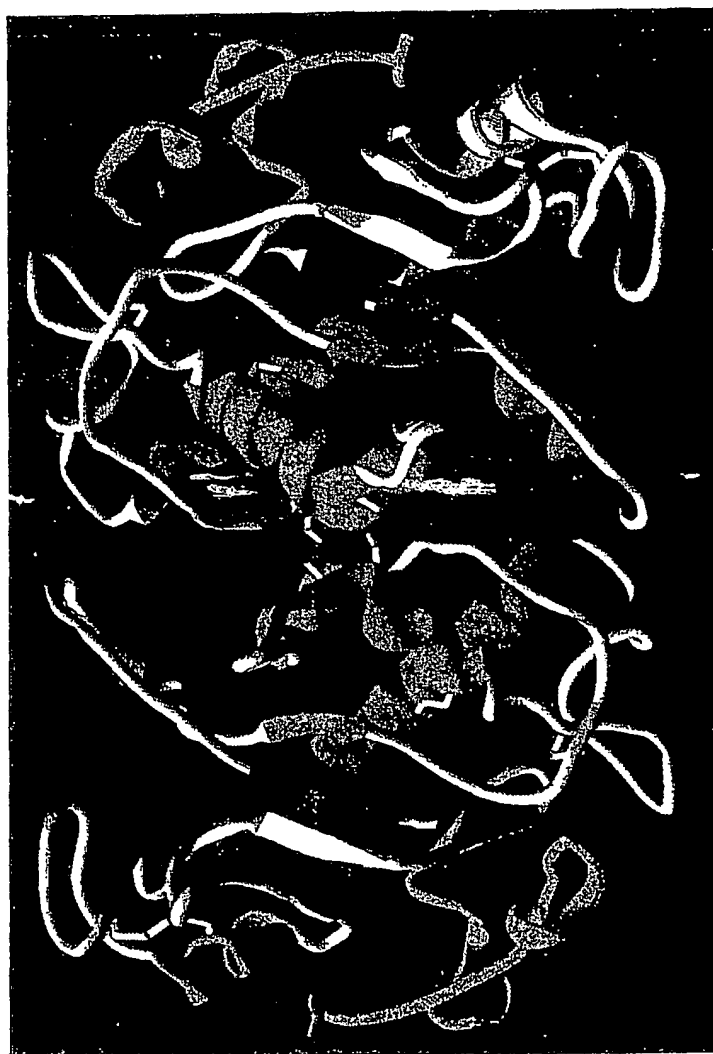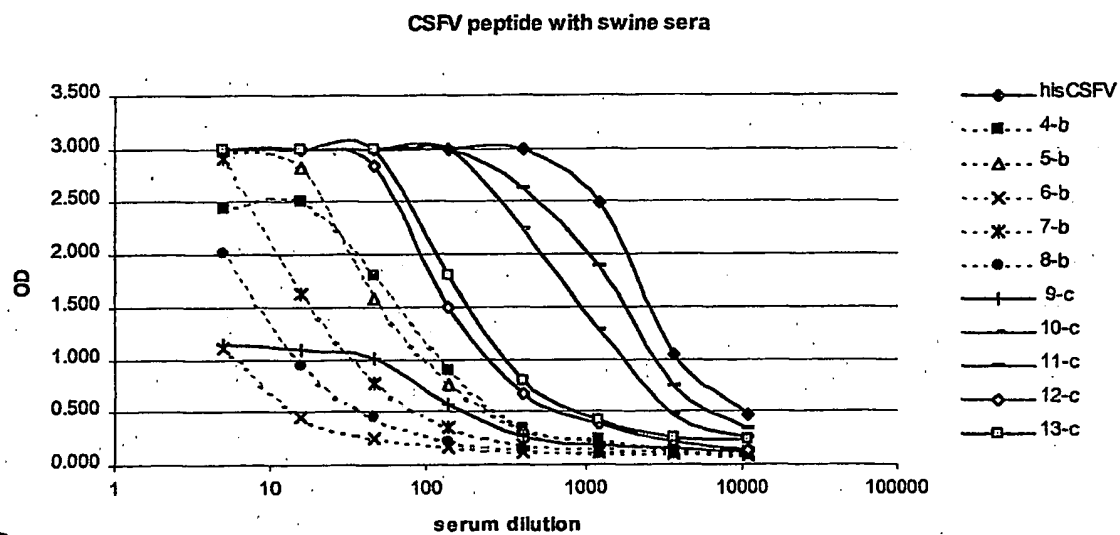Fig. 1

ENARQGAARVTSWLGRQLRIAGKRLEGRSKTWFGAYA

Fig. 2

Fig. 3

```
csfv         ENARQGAARVTSWLGRQLSTAGKRLEGR-SKTWFGAYA
bvdv         ESARQGTAKLTTWLGRQLKKLGKKLENK-SKTWFGAYA
magainin     --------GIGKFLHSAGKF-IGK-GK--I-AFVGEIMKS
restrictocin --DGNGKLIKGRTPIKFGKADCDRPPKHSQNGMK
```

Fig. 4

Fig. 5

CSFV peptide with swine sera

a

BVDV peptide with swine sera

b

Fig. 5, contd.

CSFV peptide met bovine sera

c

BVDV peptide with bovine sera

d

Fig. 6

Catching of biotinylated CSFVpeptide, blocking with BVDV peptide

a

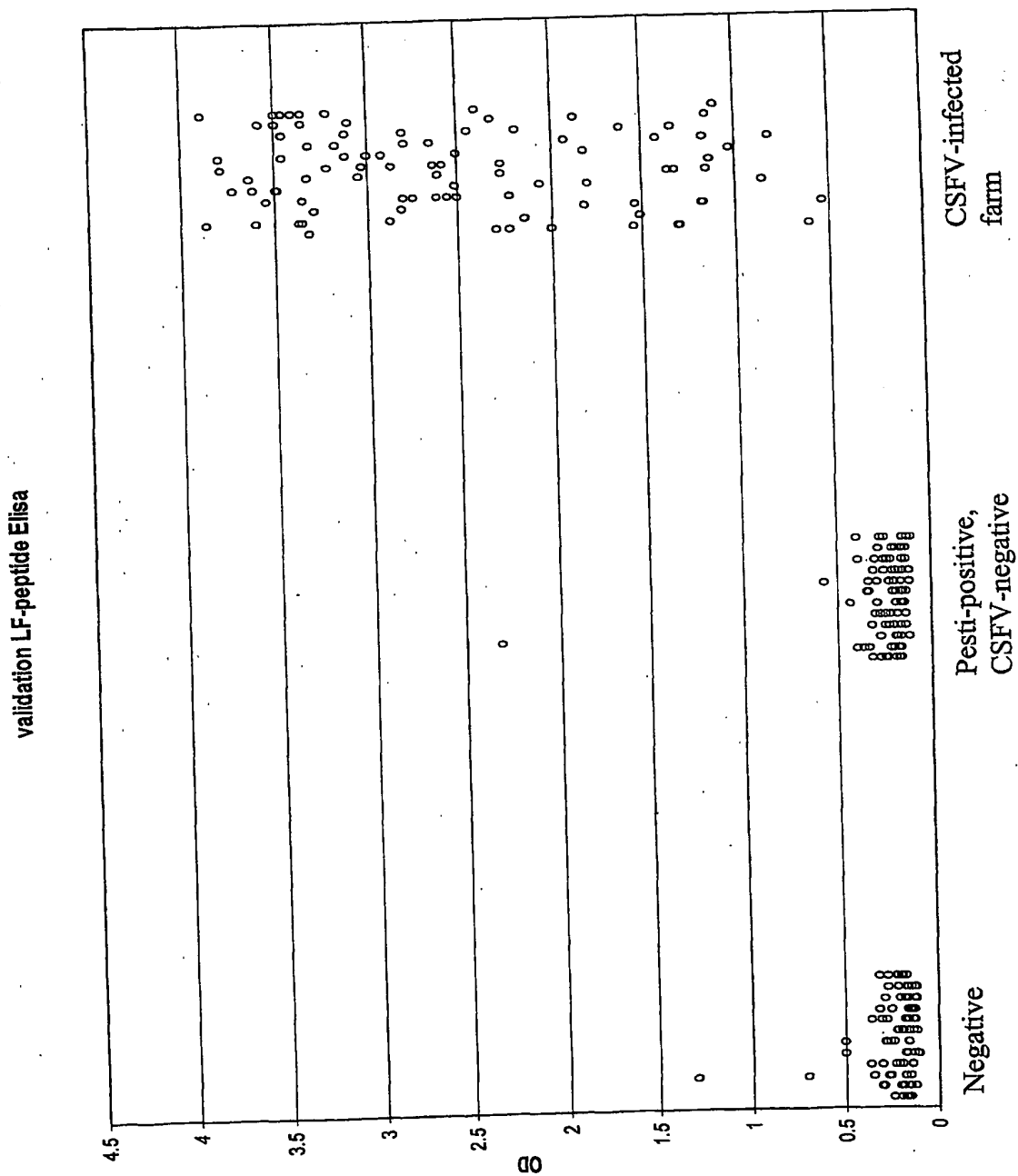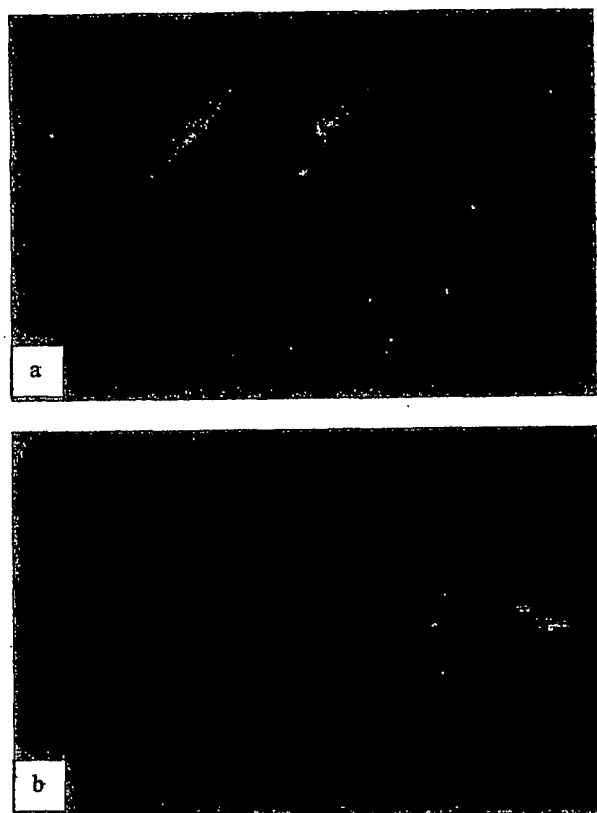Catching of biotinylated CSFV peptide, blocking with BVDV peptide

b

Fig. 7

validation LF-peptide Elisa

Fig. 8
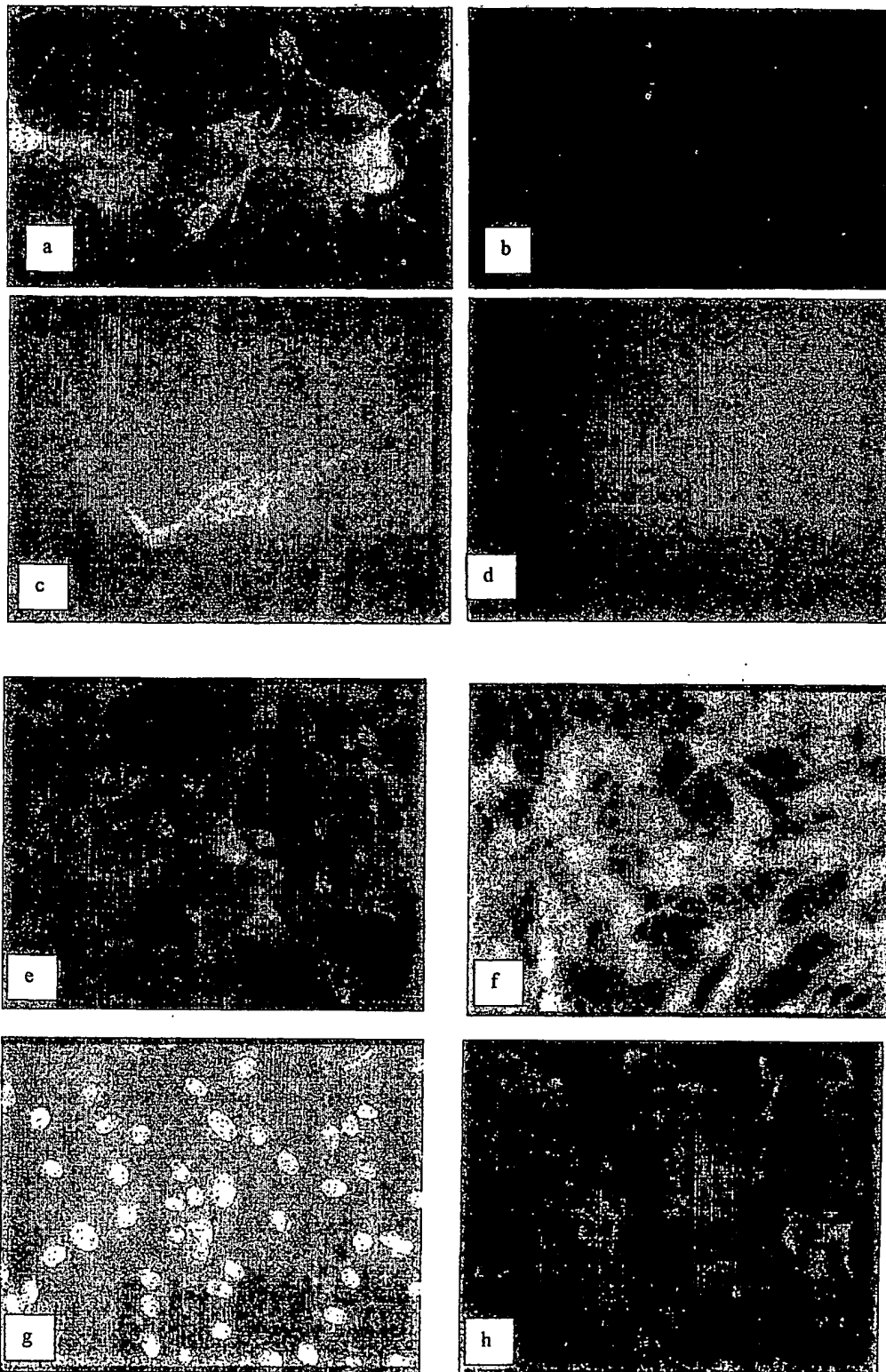
Fig. 9

Fig. 10

Fig. 11A

Fig. 11B

Fig. 12

Dose-response

legend:
- □ control
- ▲ 1:10
- ▼ 1:100
- ◆ 1:1000

y-axis: pg HRP/well (0, 50, 100, 150)
x-axis: time (min) (0, 30, 60, 90, 120, 150, 180, 210, 240)